GENE THERAPY APPROACHES FOR SOLID TUMORS: OVARIAN CARCINOMA AND BIOREDUCTIVE CHEMOTHERAPY

By

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This dissertation is dedicated to my wife, Jennifer, for being my co-pilot on this roller coaster ride.

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Abstract of Dissertation Presented to the Graduate School
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Ovarian carcinoma remains a leading cause of death due to gynecological malignancies. A new therapeutic approach currently under development for the treatment of solid tumors is gene therapy. Transgenes may be limited in expression to a particular subset of cells by placing them under the control of regulatory elements that are induced following cellular exposure to stress. Tumor cells experience stresses from such agents as hypoxia, radiotherapy, and chemotherapy.

Virally directed enzyme prodrug therapy is one strategy designed to employ a viral vector to deliver an enzyme that transforms a prodrug into a more toxic compound. The enzymology of bioreductive chemotherapy bioactivation may have the potential to be exploited in this strategy. The knowledge of the role of a particular reductase in the bioactivation of a drug allows for the design of treatment strategies aimed at targeting the cytotoxic actions of that compound.

The objectives of this work were: (1) to examine the ability to control transgene expression with the transcriptional response to cellular stress; and (2) to develop a virally directed enzyme prodrug approach based on the relationship between bioreductive chemotherapy agents and their bioactivation by cellular reductases.

Modest increases in gene expression were observed in the ovarian tumor cells only at clinically irrelevant doses following transfection of adenovirus and adeno-associated virus proviral plasmids containing the stress responsive promoters of genes induced by hypoxia, radiotherapy, and chemotherapy. The relationship between oxygenation status, NQO1 and CYPOR expression, and the cytotoxicity of MMC, EO9, and tirapazamine was examined in ovarian tumor cells to determine enzyme/drug relationships that may be exploited in an enzyme-directed manner.

A strong enzyme/drug correlation was revealed for NQO1/EO9. An enhanced cytotoxic response to EO9 was observed in those tumor cell lines that had elevated NQO1 levels. To test the ability to improve the cytotoxicity of EO9 in ovarian tumor cells that are inherently low in NQO1 expression, we delivered and overexpressed NQO1 using recombinant gene delivery systems prior to bioreductive drug administration. These cells exhibited increased sensitivity to EO9 following gene delivery. These experiments set the stage for examining the ability of improving the *in vivo* response of low NQO1

expressing human ovarian tumor xenografts to EO9 using recombinant gene delivery systems.

CHAPTER 1
INTRODUCTION

Solid tumors of various tissue types remain among the leading causes of morbidity and mortality in the United States. Currently, there are three conventional modalities for the treatment of these cancers. A cancer patient's options include surgery, radiotherapy, and chemotherapy. While surgery and radiotherapy can be effective in the treatment of some primary solid tumors, many tumor types remain resistant to therapy. Many solid tumors are inoperable or at best lend themselves only to partial surgical resection. Contributing factors to the failure of radiotherapy and chemotherapy to treat solid tumors effectively include the small therapeutic window for such highly toxic treatments, various mechanisms of inherent and/or acquired therapeutic resistance, and the abnormal microenvironments found within solid tumors. Consequently, many of the cancer patients who at the time of first diagnosis present only with primary disease will not be cured.

Therapeutic Index for Cancer Therapy

All anticancer therapies have side effects as well as antitumor effects, and toxicity to normal tissues limits the extent of the therapy that can be given to patients. The relationship between the probability of a biological effect and the treatment dose is usually described by a sigmoid curve (Figure 1-1). If a treatment is to be useful, the curve describing a probability of an antitumor effect must be displaced toward lower doses as compared to the curve describing the probability of a major toxicity. Alternatively, displacing the toxicity probability curve toward higher doses may enhance the efficacy of a treatment by protecting normal tissues. This has led to the concept of the therapeutic index (or therapeutic ratio). The therapeutic index is defined as the ratio of the doses required to produce a given probability of toxicity and antitumor effect (Figure 1-2). Any level of probability may be selected, and the appropriate endpoints of tumor response and normal tissue toxicity will depend on the limiting toxicity of the agent and the intent of the treatment. Unfortunately, even under the best of circumstances, this ratio is quite small, and in many instances significant antitumor effects are achieved only in the presence of considerable treatment side effects.

In the case of chemotherapy, suboptimal doses must often be used to avoid these unacceptable toxicities. For instance, many of the current chemotherapeutic agents target DNA and its metabolism. This approach is based on the premise that tumor cells are actively dividing and should therefore be inherently more sensitive to perturbations in the processing of DNA. While tumor cells are indeed quite sensitive to interference with DNA processing, various nonmalignant tissues also undergo active cell division, including the lining of the gastrointestinal tract and the many cell types that populate the bone marrow. Nausea and immune suppression are common side effects of the administration of these agents and, if severe enough, can lead to cessation of therapy. An improvement in the therapeutic index is therefore a main goal of experimental chemotherapy.

Therapeutic Resistance Mechanisms

Despite the fact that solid tumors are thought to arise from a single progenitor cell (Wainscott and Fey, 1990), during malignant progression, multiple additional genetic events may occur that lead to considerable heterogeneity in the biological and clinical behavior of the tumor. Solid tumors are comprised of many tumor cell subpopulations that may vary in their growth conditions, gene expression, chromosomal abnormalities, and, most critically, in their response to anticancer therapies. This cellular heterogeneity within the tumor is a major reason for employing combination treatment regimens. Such regimens could include combined radiotherapy and chemotherapy or simply a combination of different anticancer drugs. In the latter, the regimens are designed to include chemical agents with multiple mechanisms for producing their cytotoxic effects and, if possible, to possess non-overlapping normal tissue toxicities. The choice of agent with differing toxicities allows for the administration of the maximum dose of each component of the regimen. Therefore, it is hoped that, by attacking multiple cellular targets with the highest possible dose of each agent, it may be possible to kill tumor cells while avoiding normal tissue toxicity as well as the development of drug resistance.

While tumor cells may initially be sensitive to the killing effects of a specific chemical agent or combination of agents, these cells often become resistant to subsequent chemotherapy. One fundamental approach to the improvement of cancer chemotherapy is to better understand how tumor cells can acquire this resistance during the course of therapy. Generally, a theme has emerged that the mechanisms of acquired drug resistance are a result of perturbations of the pathways of cellular homeostasis. Such mechanisms include alterations in the composition of the cellular membrane (Escriba *et al.*, 1990), changes in the levels of expression and activity of membrane pumps (Ambudkar *et al.*, 1999), modified ionic environments (Theibaut, *et al.*, 1990), alterations of the levels of expression of target molecules (Hochhauser and Harris, 1993), and changes in the expression and activity of proteins necessary for drug detoxification and DNA replication and repair (Vamecq *et al.*, 1993; Hochhauser and Harris, 1993).

Interestingly, abnormalities of the tumor microenvironment have recently been implicated as contributing factors for the development of drug resistance. For instance, the presence of oxygen-deficient or hypoxic cells in solid tumors is now believed to contribute to therapy resistance either directly, due to the lack of oxygen which some chemotherapeutics and ionizing radiation require in order to be maximally cytotoxic, and/or indirectly, via altered cellular metabolism which decreases drug cytotoxicity and enhanced genetic instability which can lead to more rapid development of drug-resistant tumor cells (Chapman *et al.*, 1974; Tomida and Tsuruo, 1999; Yaun and Glazer, 1998; Reynolds *et al.*, 1996). Hence, further elucidation of the impact of the unique physiology of solid tumors on therapeutic outcome may uncover ways of improving conventional anticancer therapy.

Solid Tumor Physiology and Abnormal Microenvironment

Due to the rapid growth of solid tumors, the functional vasculature is often inadequate to supply sufficient and homogeneous support to the entire malignant cell population (Vaupel *et al.*, 1989; Vaupel, 1992; Vaupel, 1996). This results in a deficiency of oxygen and other nutrients supplied to the tumor cells, as well as the inadequate removal of cellular waste products. The failure to adequately remove these waste products results in a significant decrease in intratumoral pH (Tannock and Rotin, 1989). While these deficiencies can contribute to cell death and necrosis within the tumor (Rotin *et al.*, 1986), many cells are known to survive under marginal conditions. Therefore, solid tumors can be comprised of malignant cell subpopulations existing under differing degrees of microenvironmental stress. Indeed, it is becoming well established that most human solid tumors are heterogeneously oxygenated and contain a significant fraction of cells that are hypoxic relative to the oxygenation of normal tissues (Hockel, 1996; Vaupel, 1996). For example, the median pO_2 measured with electrodes for human breast tumors was 28 mm Hg, whereas that of normal breast was 68 mm Hg (Figure 1-3). Similar data have been obtained in squamous carcinoma of the cervix and head and neck (Nordsmark *et al.*, 1996). A review for most data available on oxygen distributions in human solid tumors demonstrates that when such tumors were compared to their normal tissue counterparts, the ratio of mean pO_2 values (normal tissue/tumor) ranged from 1.5 to 7 (Vaupel, 1996)

Models demonstrating the dynamic nature of the tumor microenvironment have been proposed (Figure 1-4). The classical view of hypoxia, and its associated acidosis, is that of chronic/diffusion-limited hypoxia (Thomlinson and Gray, 1955). Because of active cellular metabolism, oxygen typically diffuses only ~150 μm from a blood vessel in tissue. Consequently, vessel spacing is critical in most normal tissues. In contrast, large intercapillary distances found within solid tumors lead to hypoxic cells existing at the rim of the oxygen diffusion distance. Cells beyond this diffusion limit are anoxic and necrotic. Surrounding the necrotic zone are cells that are hypoxic, yet remain viable and clonogenic. In this model it is assumed that the blood vessel remains patent and that the hypoxic cells are confined to the areas near the necrosis. A second type of hypoxia that may determine the response of solid tumors to therapy is acute or perfusion-limited hypoxia (Brown, 1979; Sutherland and Franko, 1980). In the acute hypoxia model, a small blood vessel within the tumor has intermittent blood flow and this results in aberrant delivery of oxygen for a shorter duration than the chronic model.

Both forms of hypoxia have the potential to play a significant role in determining tumor response to treatment. Chronically hypoxic tumor cell subpopulation can be resistant to certain treatment modalities as the result of the diminished oxygen concentrations *per se* (radiotherapy and chemotherapy), or as a result of the reduced proliferation rate of these hypoxic cells (chemotherapy targeting DNA metabolism). In addition, these diffusion-limited hypoxic cells may be resistant to the toxic effects of a chemotherapy agent because their distance from a blood vessel prevents them from encountering the delivered chemotherapeutic agent at a sufficiently toxic dose. Acutely hypoxic tumor cell subpopulations may also impact therapeutic outcome. In the case of radiotherapy, cells irradiated when blood flow is present will behave radiobiologically as oxygenated cells. However, the blood flow may transiently stop (e.g., as a consequence of vascular collapse due to the high interstitial pressure found within solid tumors), and the existing oxygen and nutrients are rapidly exhausted. The cells that were perfused by the collapsed vessel are left in a temporarily hypoxic state. If radiation is given when the cells are not perfused, these cells behave as radiobiologically resistant hypoxic cells. From a chemotherapy point of view such cells could be resistant simply due to the inability of the drug to reach them following the collapse of the vessel. Therefore, heterogeneity in tumor oxygenation also has the potential to protect tumor cells from damage by cytotoxic therapies that are directly and/or indirectly oxygen dependent.

Investigations into the clinical impact of hypoxia within human solid tumors are ongoing. Thus far, a relationship between the presence of hypoxia and poor clinical outcome following conventional treatments has been observed in various tumor types. Indeed, studies including patients with advanced squamous cell carcinoma of the cervix, head and neck, and soft tissue sarcomas illustrate the deleterious effect of inadequate tumor oxygenation on the outcome of treatment. For instance, a study of therapeutic outcome for patients with advanced carcinoma of the cervix treated with radiotherapy and chemotherapy demonstrated that those patients whose tumors possessed low oxygen tensions had a 50% survival rate of 8 months vs. 3 years for patients with well-oxygenated tumors (Hockel *et al.*, 1993). Oxygen tension measurements in lymph node metastases of head and neck cancers demonstrate a significant relationship between low oxygen levels and failure to respond to radiotherapy (Gatenby *et al.*, 1988). Hypoxia may also play a role in determining the metastatic potential of certain tumors. For one population of patients with soft tissue sarcomas, a correlation was observed between pre-therapeutic tumor hypoxia and the development of metastasis following treatment with radiotherapy and surgery (Brizel *et al.*, 1996).

One therapeutic approach to combat the problem of inadequate oxygenation in solid tumors has been to try to increase this oxygenation prior to anticancer therapy. A number of strategies to improving tumor oxygenation are being investigated including the administration of artificial blood substitutes (Teicher and Rose, 1984; Song *et al.*, 1985; Rockwell *et al.*, 1986), right shifting of the oxyhemoglobin dissociation curve (Siemann *et al.*, 1979; Siemann and Macler, 1986; Hirst and Wood, 1987), the use of agents that increase tumor blood flow (Vaupel and Menke, 1989), and high oxygen content gas breathing either alone (Fenton and Siemann, 1995) or coupled with the agent nicotinamide (Horsman *et al.*, 1994; Siemann *et al.*,1994). Chemical sensitizers that mimic oxygen's ability to increase the sensitivity of hypoxic cells to radiotherapy and chemotherapy also aim to overcome the oxygen deficiency (Phillips *et al*., 1984). An alternative approach is to attempt to exploit this property of the tumor and, hence add a degree of tumor selectivity to the therapeutic approach. One strategy extensively evaluated in our laboratory (Siemann and Sutherland, 1992; Siemann, 1996) and a topic of this dissertation is the utilization of therapies designed specifically to attack the hypoxic cell subpopulations using chemical agents demonstrating greater bioreductive activation by hypoxic than aerobic cells (Workman, 1992; Workman and Stratford, 1993; Brown, 1996).

Bioreductive Chemotherapy and The Role of Cellular Reductases

Bioreductive chemotherapy is based on the concept that chemical agents can be designed that could specifically act as hypoxic cytotoxins. Generally, two types of these chemical agents have been considered. Agents designed to be toxic only in the absence of oxygen or agents that, while toxic to the aerobic cells, demonstrated greater activity under hypoxic conditions. Regardless of the strategy, the hope is that bioreductive metabolism, and consequent drug activation to more toxic reactive species, will occur to a greater extent in hypoxic cells than in cells that are well oxygenated. Based on evidence from the study of the aerobic and hypoxic mechanisms for the bioactivation of such agents, the concept of enzyme-directed bioreductive anticancer therapy has subsequently emerged (Workman and Walton, 1990; Workman *et al.*, 1991; Workman, 1994). In this approach, the selectivity of bioreductive chemotherapy has the potential to be governed by the oxygenation difference between tumors and normal tissue as well as the expression of the enzymes catalyzing the reductive metabolism of these agents. An important goal in the successful application of this treatment strategy is to define possible enzyme/prodrug combinations. These enzymes may be effective in this strategy if they are responsible for the aerobic and/or the hypoxic bioactivation. With this knowledge, it may also be possible to enhance the intratumoral cytotoxicity of these agents through the overexpression of these reducing enzymes within the tumor by inductive and/or genetic means prior to administration of the chemotherapy. This approach may be especially appropriate in tumors determined to be inherently low in these enzymes and/or harbor mutated forms of the enzyme rendering it nonfunctional.

Two leading classes of bioreductive agents are currently under active investigation (Rockwell *et al.*, 1993; Brown, 1993). These include the quinone antibiotics and the aromatic N-oxides (Figure 1-5).

Mitomycin C (MMC) is the prototype quinone containing bioreductive chemotherapeutic agent (Sartorelli *et al.*, 1994), and is currently used clinically in the treatment of solid tumors. Various one- and two-electron bioreductive enzymes have been implicated in the bioactivation of MMC under both aerobic and hypoxic conditions (Figure 1-6) (Spanswick *et al.*, 1998). The main difference between the one-electron and two-electron reductions lies in the possibility for one-electron reductases to redox cycle in the presence of oxygen. In the process of redox cycling (Kappus, 1986), the quinone is reduced to form a semiquinone that then undergoes a reaction with molecular oxygen, producing a superoxide radical and restoring the original quinone molecule. These superoxide radicals and their secondary radicals (e.g. hydroxyl radical) can cause a variety of lesions in DNA. Two-electron reduction of a quinone leads to formation of the hydroquinone, bypassing the semiquinone and possible redox cycling. It has been observed that MMC can be metabolized by enzymes such as DT-diaphorase (NQO1) (Siegel *et al.*, 1992), NADPH: cytochrome P450 reductase (CYPOR) (Bligh *et al.*, 1990), NADH: cytochrome b5 reductase (Hodnick and Sartorelli, 1993), xanthine oxidase (Pan *et al.*, 1984), and xanthine dehydrogenase (Gustafson and Pristos, 1992). Recently, a novel mitochondrial reductase has been identified that, under hypoxic conditions, more effectively metabolizes MMC than all others yet investigated (Spanswick *et al.*, 1996). To date, it appears that under different physiological conditions certain reductases predominate over others and the determination of the enzymes that are most important for the killing effects of MMC remain unequivocal.

The metabolism of an analogue of MMC, the indoloquinone, EO9 (3-hydroxymethyl-5-aziridinyl-1-methyl-2- (1H-indole-4, 7-dione)-prop-β-en-α-ol) is somewhat clearer (Oostveen *et al.*, 1987). This compound was designed so that the reduction of the quinone moiety of EO9 would induce the release of the hydroxyl groups at the C-1 and C-10 carbon atoms, as well as the opening of the aziridine ring, generating at least three reactive centers. For EO9, it has been suggested that under hypoxic conditions one-electron transfer is dominating, and, in air, both processes take place. Xanthine oxidase is a one-electron reductase that may play a role in the bioactivation of EO9 (Maliepaard *et al.*, 1995). It was observed that the reductive activation of EO9 by purified xanthine oxidase produced DNA cross-links under hypoxic conditions. In addition, this study observed the formation of these cross-links occurs following the metabolism of EO9 by purified rat NQO1. Subsequently, the overexpression of human NQO1 in Chinese hamster ovary cells was observed to lead to an increase in the aerobic sensitivity of these cells to EO9 (Gustafson *et al.*, 1996). Likewise, induction of NQO1 by pretreatment of L5178Y murine lymphoma cells with the 1,2-dithiole-3-thione, oltipraz, leads to an increase in the aerobic toxicity of EO9 (Begleiter *et al.*, 1996; Doherty *et al.*, 1998). Interestingly, this relationship is also observed when comparing the high NQO1 expressing colon carcinoma cell line HT-29 to the colon carcinoma cell line BE that has no detectable NQO1 (Traver *et al.*, 1992). The BE cell line's lack of NQO1 activity is due to a point mutation at position 609 in the NQO1 gene. It is believed that this inactivity is due to inefficient translation of the mutant NQO1 transcript and/or rapid degradation of the mutant NQO1 protein, since this cell line has abundant NQO1 mRNA. Other aziridinyl benzoquinones such as diaziquone, methyldiaziquone, and the hydroxyl radical generating quinone, streptonigrin, also have been determined to be bioactivated by NQO1 (Gustafson *et al.*, 1996).

Another chemical class of bioreductive chemotherapy is the aromatic N-oxides. Its lead compound, SR4233 (3-amino-1, 2,4-benzotriazine 1,4-

dioxide; tirapazamine) is a potent hypoxic cytotoxin that elicits high aerobic/hypoxic differentials in many tumor cell types (Brown, 1993). The proposed mechanism (Figure 1-7) for its impressive hypoxia specific cytotoxicity is that following the reductive metabolism of the compound by one-electron reductase(s) a radical anion is formed. The reduced tirapazamine may be back oxidized to the parent drug in the presence of oxygen, generating superoxide radicals, and the subsequent secondary radicals. In the absence of oxygen, the reduced tirapazamine, after protonation, abstracts hydrogen from DNA, leading to single and double strand breaks. Unlike the quinones, it appears that one-electron reduction of tirapazamine is involved in the bioactivation, while two-electron reduction results in detoxification of the agent. One-electron reductases such as cytochrome P450 (various isozymes) (Fitzsimmons *et al.*, 1994; Walton *et al.*, 1992), CYPOR (Walton *et al.*, 1992), and xanthine oxidase (Laderoute *et al.*, 1988; Wang, 1993) have been observed to metabolize tirapazamine to a more toxic species. For example, direct activation of tirapazamine by purified rat CYPOR has been observed to result in the formation of single- and double-strand breaks in DNA (Laderoute *et al.*, 1988). In addition, the relationship between CYPOR activity and sensitivity to tirapazamine was examined in a panel of breast cancer cell lines (Patterson *et al.*, 1995), and a strong correlation was revealed under both aerobic and hypoxic conditions. Furthermore, this group has demonstrated that the stable overexpression of CYPOR in one of these breast lines correlates with an increase in the aerobic and hypoxic sensitivity of these cells to tirapazamine

(Patterson *et al.*, 1997). More recently it has been discovered that nuclear reductases also play an important role in the bioactivation of this compound (Evans *et al.*, 1998). Two-electron reduction by NQO1 has been observed to detoxify tirapazamine (Patterson *et al.*, 1994). Taken together, the above evidence suggests that a CYPOR/tirapazamine combination may have the potential to be exploited in an enzyme-directed treatment scenario.

To date, NQO1 has generated the most interest of the potential bioactivating enzymes because it is overexpressed in many tumors and tumor cell lines (Ross *et al.*, 1994; Workman, 1994). The enzyme is an obligate two-electron reductase, which has no preference for NADH or NADPH as an electron donor, and can be inhibited by low concentrations of dicoumarol (Ernster, 1967). Physiologically, NQO1 has two main functions: the reduction of precursors of vitamin K3 important for biosynthesis of prothrombin and related blood clotting factors, and it serves a protective function as a detoxifying Phase I/II metabolizing enzyme.

Elevated levels of NQO1 have been observed in tumors and tumor cell lines from human lung, liver, colon, breast, and brain (Schlager and Powis, 1990; Malkinson *et al.*, 1992; Cresteil and Jaiswal, 1991; Workman *et al.*, 1991; Berger *et al.*, 1985). However, other tumor types, such as human kidney and stomach, have been found to possess decreased NQO1 activity relative to normal tissue (Schlager and Powis, 1990). Thus, while increased expression of NQO1 is generally seen in tumor tissue, there is a marked variation in this expression both intra- and intertumorally.

Gene Therapy Approaches to Cancer Therapy

A new therapeutic approach for the treatment of solid tumors currently under active development is gene therapy (Roth and Cristiano, 1997). In recent years, many novel gene therapeutic strategies have been proposed. One strategy is to target the tumor cell's underlying genetic lesion. This may be accomplished by inactivating the expression of an oncogene and/or through the replacement of an inactive tumor suppressor gene. For instance, targeting of the *ras* oncogene with antisense (Kita *et al.*, 1999) or ribozyme technologies (Giannini *et al.*, 1999) have been observed to inhibit tumor cell growth. Likewise, replacement of a mutant p53 tumor suppressor with a normal protein inhibits tumor growth (Gallagher and Brown, 1999). Other strategies that have shown promise involve the augmentation of immunotherapeutic and chemotherapeutic approaches. These strategies include *ex vivo* and *in vivo* cytokine gene transfer (Simons and Mikhak, 1998; Leroy *et al.*, 1998), the use of drug resistance genes for bone marrow protection from high-dose chemotherapy (Licht *et al.*, 1995), and drug sensitization with genes responsible for the bioactivation of prodrugs (enzyme/prodrug) (Singhal and Kaiser, 1998). The latter approach may provide opportunities for more selective targeting of the toxic effects of chemotherapeutic agents to solid tumors. The underlying principle of this approach is to deliver to the tumor a gene encoding an enzyme that has the ability to transform a nontoxic (or less toxic) prodrug into a toxic compound. Thus, the cells bearing the "suicide gene" are killed when the prodrug is administered. Current "suicide genes" under investigation mediate sensitivity by encoding viral, bacterial, or mammalian enzymes. The herpes simplex virus thymidine kinase/ganciclovir system is the most extensively studied of such two-stage systems (Moolten, 1986). Other enzyme/prodrug systems under investigation include the *E. Coli* cytosine deaminase gene/5-fluorocytosine system (Huber *et al.*, 1993) and the mammalian cytochrome p450 2B1 gene/cyclophosphamide system (Wei *et al.*, 1994). To optimize the selectivity of this enzyme/prodrug approach these genes must be delivered to the tumor cells while limiting expression in surrounding normal tissue.

Despite the proliferation of clinical protocols using gene therapy to treat solid tumors, there are many aspects of gene transfer that are less than ideal. Critical to the success of gene therapy are the efficient transfer of the chosen transgene to the target cell and the controlled regulation of that transgene once delivered. One of the most important areas for future research is vector design. The vector is critical for gene delivery and expression of the transgene, but existing vectors all have limitations. These limitations include the requirement of a direct intratumoral injection in order to deliver the vector, and the expression of the transgene will be limited to only a subpopulation of the tumor cells.

One way in which transgenes may be delivered to solid tumors is by employing viral vectors. Adenovirus and adeno-associated virus (AAV) are two such vectors currently under development (Kremer and Perricaudet, 1995). Since adenovirus can transduce a large variety of human cell types, it is being explored as a vector to deliver genes to tumor cells. The major advantages of adenoviral vectors are that they can be produced at very high titers and can be used for gene delivery *in vivo.* The wild type genome is ~36 kb in size, and the early E1A, E1B, and E3 genes are normally removed to produce a vector. Thus, approximately 7.5 kb of sequence is available for insertion of transgenes of interest. This vector is currently being studied in a wide variety of genetic approaches to cancer therapy, including enzyme/prodrug scenarios (Blackburn *et al.*, 1999). Since adenoviruses rarely integrate into the host cell genome, gene expression will be transient. This may make redelivery of the vector necessary. The existing host immune response to adenoviral proteins in the vector itself, or produced by the transduced cells, may increase the toxicity and/or limit the efficacy of any reintroduction of the recombinant adenoviral vector.

An alternative approach would be to employ recombinant AAV vectors for gene delivery. AAV is a relatively new member of the current viral gene delivery systems (Muzyczka, 1992). One advantage of this type of vector is that AAV is a defective parvovirus that requires coinfection by a second unrelated helper virus, such as adenovirus or herpes virus, for productive infection. In the absence of helper virus infection, AAV remains latent within the cell by preferentially integrating into a site on human chromosome 19. The viral genome contains two open reading frames: the *rep* gene, which encodes the proteins necessary for viral replication and integration, and the *cap* gene for the structural capsid proteins. These genes are flanked by two short inverted terminal repeats (TR) which are the only genomic elements required *in cis* for replication, integration, and packaging. To date, studies of AAV's application to cancer gene therapy have been limited. Yet AAV has several characteristics that may be advantageous to the treatment of solid tumors. These include its lack of any known pathogenicity, the ability of these vectors to carry cellular transcriptional control elements for true tissue specific or inducible expression of a therapeutic gene without interference from the remaining viral sequences, and high transduction frequencies in both dividing and nondividing cell populations. Two significant disadvantages of the use of AAV are the limited packaging capacity of the virus (~5kB), and the difficulty in the production and purification of recombinant AAV of consistently high titer for use in gene therapy studies.

The regulated expression of the virally delivered transgene is another important aspect for the development of gene therapy that is selective for solid tumors (Miller and Whelan, 1997). Through the use of specific regulatory elements that control gene expression within the tumor, it may be possible to target gene expression of virally delivered transgenes intratumorally. One strategy is the employment of tumor specific promoters that are based on the gene expression of the normal tissue from which the tumor has arisen. For instance, melanocyte-specific promoters have been used for targeting gene expression in melanoma cells following systemic and localized administration of gene based therapeutics (Vile and Hart, 1993; Vile *et al.*, 1994). An alternative approach is to employ regulatory elements specific for proteins expressed by some tumor types that are normally only found during fetal development. The promoter of the alpha-fetoprotein gene, which is highly expressed in hepatomas, is one such element (Ishikawa *et al.*, 1999). Finally, the expression of the therapeutic gene may be placed under the control of an inducible promoter. A number of genes have been identified that are induced following cellular exposure to stressful environmental conditions. Nongenotoxic cellular stress, such as the deprivation of oxygen, has been shown to lead to the expression of specific genes as the cells attempt to adapt. Specific hypoxic stress response proteins have now been identified and include erythropoietin and vascular endothelial growth factor (Zhu and Bunn, 1999). The regulatory regions of these hypoxia responsive genes have the potential to be used in gene delivery systems in order to confine the expression of the transgene to the hypoxic tumor cell subpopulation. Hence, the abnormal tumor microenvironments may provide an opportunity for further tumor targeting through transcriptional regulation of the delivered transgene (Dachs *et al.*, 1997). In addition, the genotoxic cellular stress response has the potential to be exploited in various gene therapy scenarios. The treatment of tumor cells with ionizing radiation and chemical agents have been observed to lead to increased transactivation from a number of promoters. By employing these promoters it may be possible to enhance the expression of delivered transgenes using the conventional treatment modalities themselves. Indeed, the ability to direct the expression of tumor necrosis factor alpha using the promoter region from the radiation inducible Egr-1 gene has been demonstrated (Hallahan *et* *al.*, 1995). This approach allows for the spatial and temporal control of gene expression during the course of radiotherapy. Similarly, the ability of the MDR1 promoter to direct the expression of a transgene following the administration of certain chemotherapy agents is being investigated (Walther *et al.*, 1997). Collectively, these approaches illustrate the potential of using transcriptional specificity to achieve selective tumor cell killing and/or normal tissue protection. Such regulation of gene expression may prove most useful following systemic delivery of the viral vector.

Rationale

The most important goal in improving the response of solid tumors to treatment is to better target the therapy. By identifying differences between normal and malignant tissues it is possible to design therapeutic strategies that exploit such differences and, hence, improve the therapeutic index. The abnormal tumor microenvironment is one major difference. The poor oxygenation of solid tumors lends itself to exploitation through targeted chemotherapy and/or through transcriptional targeting of gene delivery systems. Transcriptional targeting of the gene delivery systems to tumor cells experiencing the stress of conventional radiotherapy and chemotherapy also holds potential for improving gene based approaches. In addition to identifying inherent differences in normal and malignant tissues, it is possible to create differences in gene expression (e.g. enzymes) between the tumor and the surrounding normal tissue through the use of recombinant viral gene delivery systems.

The central goal of this project was to develop and evaluate new gene therapy strategies for the treatment of solid tumors. Its specific aims are:

Specific Aim 1: To evaluate the potential of using the promoter regions from genes that are induced by the tumor microenvironment or conventional anticancer therapies in adenovirus and AAV proviral plasmids as a means of transcriptionally targeting gene constructs to solid tumors.

Specific Aim 2*:* To construct and evaluate novel adenovirus and AAV proviral plasmids in which one CMV promoter controls the expression of a bioreductive enzyme and the reporter gene, GFP.

Specific Aim 3: To examine the relationship between the efficacy of 3 bioreductive anticancer agents and their metabolism by cellular reductases in order to establish enzyme/prodrug combinations for employment in virally directed enzyme/prodrug therapy for tumors.

Specific Aim 4: To employ a specific bioreductive enzyme/prodrug combination to further sensitize tumor cells to the killing effect of bioreductive chemotherapy using adenovirus and adeno-associated virus delivery systems.

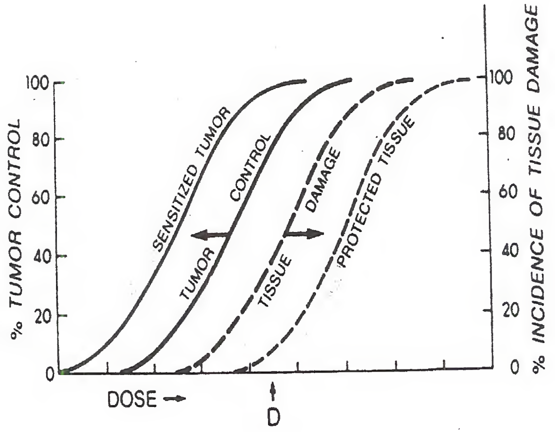


Figure 1-1. Relationship between the probability of an antitumor response or normal tissue damage and the dose of an anticancer treatment modality, and strategies for improving treatment. Improvements in the overall effectiveness of a treatment may be accomplished by shifting the tumor response curve (solid line) to lower doses and/or shifting the normal tissue damage curve (dashed line) to higher doses (Rubin and Siemann, 1993)

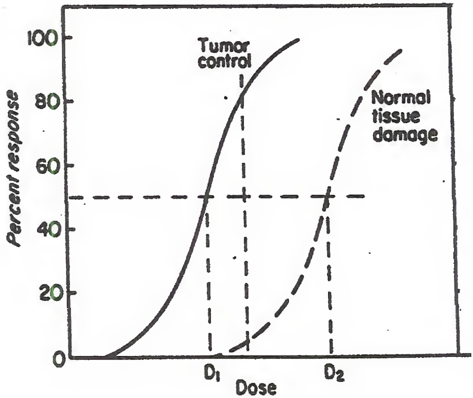


Figure 1-2. Calculation of the therapeutic index for cancer therapy. D1=dose at which treatment elicits 50% tumor response; D2=dose at which treatment elicits 50% normal tissue damage. Therapeutic index=D2: D1 (Rubin and Siemann, 1993, with modification).

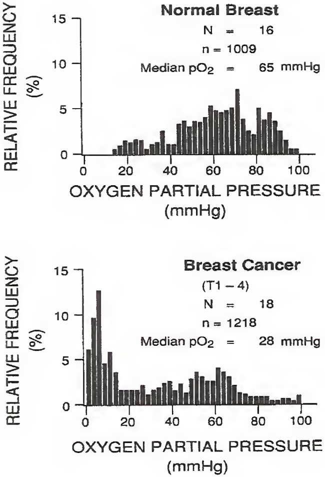


Figure 1-3. Oxygen partial pressure distribution for normal breast and breast cancer (clinical stages T1-4). N=number of patients; n=number of oxygen partial pressure measurements made with needle electrodes using computerized oxygen partial pressure histography (Vaupel, 1994).

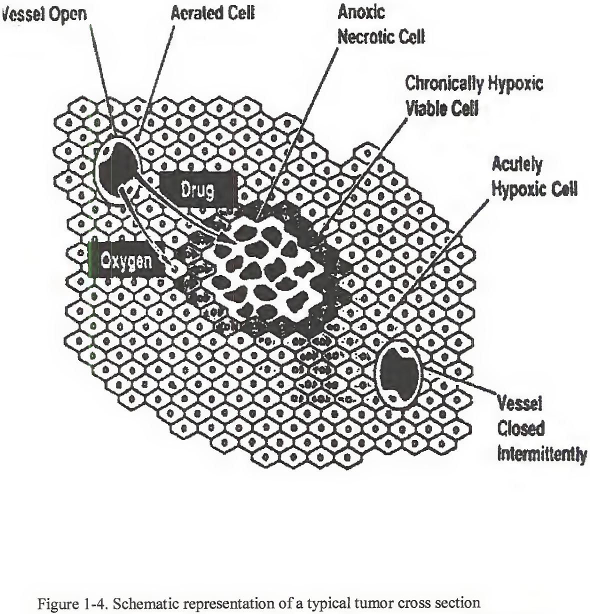
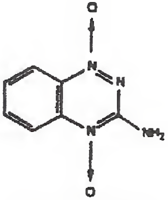
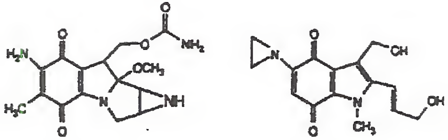


Figure 1-4. Schematic representation of a typical tumor cross section illustrating the two basic models for the development of hypoxia in solid tumors (Siemann, 1992).



tirapazamine



mitomycin C

EO9

Figure 1-5. Structures of N-oxide (tirapazamine) and quinone antibiotic (mitomycin C, EO9) bioreductive anticancer agents.

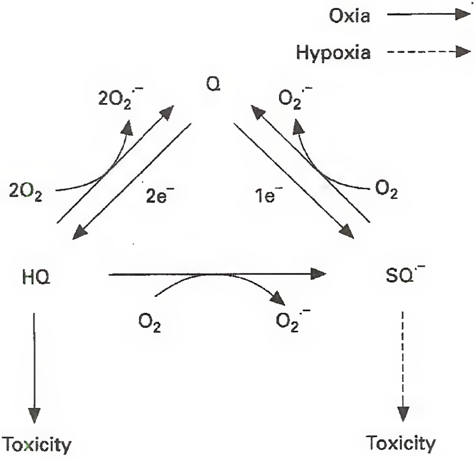


Figure 1-6. Bioactivation of quinones under aerobic and hypoxic conditions. Q=quinone; HQ=hydroquinone; SQ=semiquinone (Ross, *et al.*, 1996).

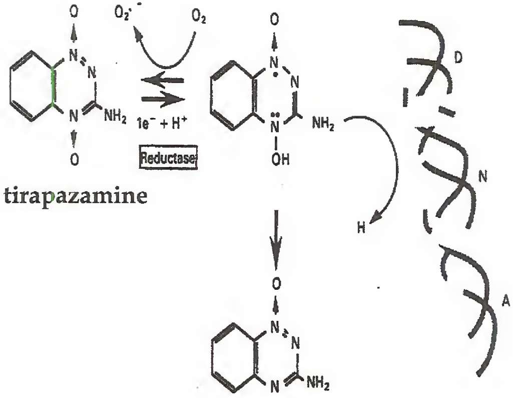


Figure 1-7. Proposed mechanism of action for the cytotoxic effects of the N-oxide bioreductive anticancer agent, tirapazamine (Brown, 1993).

CHAPTER 2

CONSTRUCTION OF ADENO- AND ADENO-ASSOCIATED PROVIRAL EXPRESSION PLASMIDS FOR CANCER GENE THERAPY

Introduction

The development of genetic approaches for cancer treatment may provide additional ways to selectively target the therapy to the tumor. Numerous vector-targeting strategies have been proposed. The gene delivery system may be targeted by physical means. The route, by which the vector is administered, such as the direct intratumoral injection of a vector, can offer a degree of selectivity to a gene therapy approach for cancer. Another approach has been to exploit the natural tropism of a viral gene delivery system. Certain viruses exhibit natural tropism for tissues that could be used to target gene delivery to specific tumor types. Such a tumor targeting strategy could be based on the presence of a specific virus receptor on a given malignant tissue or based on the fact that the tumor cells are rapidly dividing. For example, herpes viruses is known to be well suited for delivery of transgenes to tumors of neuronal origin (Kanno, *et al.*, 1999). In addition, mutant forms of wild type viruses have been observed to have malignant cell tropism. The E1B 55K adenovirus can preferentially replicate and kill cells that are rapidly dividing and have become malignant as a result of a p53 deficiency (Rothmann, *et al.*, 1998). The tropism of viral vectors also may be engineered to target various tumor types by altering the capsid proteins so that the virus possesses new cellular receptor recognition sites (Cosset and Russell, 1996). The regulated expression of gene delivery systems for targeting cancer gene therapy to solid tumors also is an area of active investigation (Miller and Whelan, 1997).

Numerous investigations into the gene therapy of solid tumors also have focused on the establishment of cell type specific or inducible expression vectors that may allow the targeted and regulated expression of therapeutic genes. The targeting of gene delivery systems to tumors through the use of *cis*-acting promoter/enhancer elements containing binding sites for tissue- or tumor-specific transcription factors have been demonstrated. Investigations into the utility of this approach have included the PSA (Goto *et al.*, 1998), CEA (Cao *et al.*, 1999), and erbB2 (Harris *et al.*, 1994) promoters for prostate, colon, and breast tumors, respectively. In addition, synthetic promoters are becoming available that can be activated/repressed by the administration of exogenous agents. One example of such a regulatory system is the tet ON/OFF system (Gossen *et al.*, 1995). In this system, gene expression can be induced or restricted following the administration of the antibiotic tetracycline. In addition, inducible control of gene expression has also been demonstrated based on the cellular response to conventional anticancer agents (Hallanhan *et al.*, 1995; Walther *et al.*, 1997).

The tumor cell response to environmental stress offers an opportunity for regulation of delivered transgenes. The aberrant solid tumor physiology as well as the treatment of solid tumors with chemotherapy and radiotherapy results in transcriptionally active tumor cell stress responses. The transcriptional targeting of gene delivery systems to the hypoxic regions of solid tumors has been proposed (Dachs *et al.*, 1997). Hypoxic induction of a number of physiologically relevant genes including those coding for erythropoietin, tyrosine hydroxylase, and vascular endothelial growth factor (Zhu and Bunn, 1999) have been reported. A number of transcriptional regulators are influenced by hypoxic stress on tumor cells, including the NF kappa B (Koong *et al.*, 1994) and HIF-1 (Blancher and Harris, 1998) transcription factors. A 385 bp fragment of VEGF promoter region containing a binding site for HIF-1 has been shown to increase expression of a luciferase reporter gene in tumor cells following exposure to hypoxia, and this induction was shown to be enhanced by the presence of a mutant *ras* oncogene (Mazure *et al.*, 1996). In order to characterize the transcriptional activation capacity of this VEGF promoter fragment in an adenovirus and AAV background, we constructed proviral plasmids containing this fragment upstream of the GFP reporter gene.

Conventional cancer treatment modalities also may be used for regulating transgene expression within the tumor. Treatment of tumor cells with radiotherapy and chemotherapy elicits a cellular stress response that involves the induction of many genes. The cellular response to ionizing radiation involves the induction of such genes as Egr-1 (Hallahan *et al.*, 1995) and TNFα (Hallahan *et al.*, 1989). Like the hypoxic stress response, the transcription factor NF kappa B is induced in tumor cells following radiotherapy (Fuks *et al.*, 1993). The use of the promoter region of the Egr-1 gene to drive transgene expression has been demonstrated following exposure of tumor cells to ionizing radiation (Hallahan *et al.*, 1995). Such an approach allows for the spatial and temporal control of transgene expression during the course of radiotherapy. The promoter region of the TNFα gene may have the potential to be similarly employed. Alternatively, the cellular stress response following exposure to clinically active chemical anticancer agents may hold potential for employment in a gene targeting approach for solid tumors. Indeed, the promoter region of the MDR-1 gene has been observed to drive the expression of the chemosensitizing cytokine TNF α following exposure of tumor cells to chemical agents (Walther *et al.*, 1997). Another gene that is induced following exposure of tumor cells to various DNA-damaging chemical agents is the Gadd153 gene and a fragment of its promoter has been shown to be highly induced by many of the conventional alkylating chemotherapeutic agents including the bioreductive agent mitomycin C (Beard *et al.*, 1996). The promoter region of this gene may have the potential to be employed in a scenario similar to that with the MDR-1 promoter. In order to characterize the radiation-inducible transcriptional activation capacity of fragments of the Egr-1 and TNF α promoters in an adenovirus and AAV background, we constructed proviral plasmids containing these fragments upstream of the GFP reporter gene (Kain *et al.*, 1995). In addition, to characterize the chemotherapy-inducible transcriptional activation capacity of a fragment of the

Gadd 153 promoter in an adenovirus and AAV background, we constructed proviral plasmids containing this fragment upstream of the GFP reporter gene.

The tumor-selectivity of a gene-based approach to cancer therapy also may be a result of the type of delivered transgene. For instance, the delivery of wild type p53 would affect only those cells carrying mutant protein (Gallagher and Brown, 1999). Alternatively, gene based approaches may also gain selectivity if the protein delivered to the tumor cells is an enzyme that also is nontoxic, but that may activate a subsequently administered chemotherapeutic agent (Singhal and Kaiser, 1998). NQO1 is an obligate two-electron reductase that has generated much interest in the bioactivation of quinone-based bioreductive anticancer agents (Worman, 1994). Intratumoral overexpression of this enzyme in tumor cells inherently low in NQO1, or in those tumor cells harboring a mutant nonfunctional form of NQO1, may improve the sensitivity of these tumor cells to quinone-based anticancer agents such as EO9. In order to test the ability to coordinately express the bioreductive enzyme NQO1 and the reporter gene GFP in a single dicistronic unit under the control of a single CMV promoter, we constructed adenovirus and AAV proviral plasmids containing such an expression cassette and transduced tumor cells inherently low NQO1.

The purpose of the present study was to determine if the transcriptional capacity of such proviral plasmids would warrant the development of recombinant gene delivery systems possessing such expression cassettes for further study in the targeting of gene-based therapeutics to solid tumors. These investigations would set the stage for future experiments aimed at utilizing recombinant viral vectors possessing such regulatory/expression cassettes for the treatment of solid tumors.

Materials and Methods

Cell Lines

Human ovarian tumor cells (SAU) were grown in DMEM medium supplemented with 10% fetal calf serum (FCS) at 37°C and 5% CO_2. Rodent KHT/iv cells were grown in α-MEM medium supplemented with 10% FCS at 37°C and 5% CO_2.

Plasmid Construction and Transient Transfections

The adenovirus proviral plasmids were constructed from pTRUF7 (Figure 2-1a). This construct contains a CMV promoter controlling expression of GFP, a single lox P site for use in *Cre*-recombinase production of recombinant virus, the neomycin resistance gene under control of a fragment of the herpes simplex thymidine kinase promoter, and the entire expression cassette is flanked by adenovirus ITRs. The AAV proviral plasmids were constructed from pTRUF5 (Figure 2-1b). This construct contains a CMV promoter controlling expression of GFP, the neomycin resistance gene under control of a fragment of the herpes simplex thymidine kinase promoter, and the AAV TRs flanks the entire expression cassette. The pVEGF-UF7 and pVEGF-UF5 plasmids were constructed by substituting a 385 bp fragment of the human VEGF promoter region for the CMV promoter in pTRUF7 and pTRUF5, respectively (Figure 2-2). The pEgr-1-UF7 and pEgr-1-UF5 plasmids were constructed by substituting a 425 bp fragment of the human Egr-1 promoter region for the CMV promoter in pTRUF7 and pTRUF5, respectively (Figure 2-3). The pTNF-UF7 and pTNF-UF5 plasmids were constructed by substituting an 1100 bp fragment of the human TNF α promoter region for the CMV promoter in pTRUF7 and pTRUF5, respectively (Figure 2-4). The pGadd153-UF7 and pGadd153-UF5 plasmids were constructed by substituting a 1045 bp piece of the human Gadd 153 promoter region for the CMV promoter in pTRUF7 and pTRUF5, respectively (Figure 2-5). The pTRUF7-NQO1 and pTRUF5-NQO1 plasmids were constructed by inserting a dicistronic unit containing the human NQO1 cDNA and GFP cDNA separated by a poliovirus type 1 IRES element downstream of the CMV promoter (Figure 2-6).

These plasmids were transfected into the tumor cell lines by liposome-mediated transfection with Lipofectamine Plus (GIBCO/BRL) using the manufacturer's guidelines. Briefly, cells were plated for 24 hr in 6-well dishes until they reached 50-70% confluence. The liposome-DNA complexes then were added to the cells in serum-free medium and incubated for 5 hr at 37°C. This mixture was removed and replaced with fresh complete medium, and the cells were incubated at 37°C for an additional 19 hr. At this time the cells were either lysed or fixed for FACS analysis.

Environmental Stresses

Twenty-four hours following transfection with the stress induced promoter constructs, the SAU cell line was exposed to the various environmental stresses. For the hypoxia induced promoter constructs, the tumor cells were placed in an airtight chamber and subjected to repeated rounds of evacuation and replacement with nitrogen gas. The sealed chambers were then incubated for various times at 37 °C and the cells were fixed for FACS analysis. For the radiation inducible constructs, the transfected cells were treated with various doses of ionizing radiation and were fixed for FACS analysis 24 hours post-irradiation. For the chemotherapy inducible constructs, the transfected cells were treated with various doses of MMC for 24 hours and were fixed for FACS analysis.

Preparation of Whole Cell Extracts and Western Blotting

Extracts were made 24 hr from the start of transfection. Extracts were prepared using an extraction buffer containing 50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% NP40 (v/v), 1 mM PMSF, aprotinin, and leupeptin (EBC buffer). Cells were washed with cold PBS, resuspended in EBC buffer, incubated at room temperature on a rocking platform for 10 min, centrifuged at 1100 rpm for 10 min, and the supernatant was saved. Protein determinations (for gel loading) were made on the extracts by Bradford Assay (BIO-RAD). Thirty μg of total cellular protein were subject to SDS-PAGE (12% polyacrylamide). Proteins were transferred from the gel to nitrocellulose membranes at 300 mA at 4°C for 3 hr. The membranes were washed with Tris-buffered saline (TBS) and blocked overnight with 5% dry milk in TBS solution at 4°C. The membranes were then incubated for 1 hr at room temperature with 5 ml of hybridoma supernatant containing a mouse anti-human NQO1 monoclonal antibody. The membranes were then washed with TBS for 30 min, and incubated with a 1:5000 dilution of a goat anti-mouse IgG conjugated to HRP (Promega, Madison, WI) for 30 min at room temperature. The blots were developed using chemiluminescence and the quantity of protein was determined using densitometry.

Preparation of Sonicates and NQO1 Activity Measurements

Following transfection, the tumor cells were washed with PBS, trypsinized, resuspended in 0.25 M sucrose in 25 mM Tris-HCl (pH 7.4), and sonicated on ice for 25 sec. The sonicates were centrifuged at 12,000 rpm for 15 min and the supernatant was saved. NQO1 activity in the supernatant was determined spectrophotometrically by following the reduction of cytochrome c at 550 nm. A 5 μl sample of the supernatant was added to the reaction mixture that contained cytochrome c (77μM), menadione (20 μM), NADPH (2 mM) as cofactor and BSA (0.14% w/v). Reactions were performed at 37° C in 1 ml Tris-HCl buffer (25 mM, pH 7.4) in the presence and absence of dicoumarol (100 μM). NQO1 activity was taken as the activity that could be inhibited by dicoumarol.

Fixation of Cells and FACS Analysis

Cells were fixed in cold 1% p-formaldehyde in PBS. The fixed cells were analyzed by FACS on a Becton Dickinson flow cytometer made available through the University Core Facility for Flow Cytometry at the University of Florida. Dead cells and debris were excluded from the analysis based on forward angle and side scatter light gating. A gate was set on the untransfected sample (M1) and cells whose fluorescence increased due to GFP expression were scored as positive if their fluorescence increase shifted them into a second gate (M2).

Results

Hypoxia Inducible Promoter: VEGF

To test the ability of a fragment of the VEGF promoter region to control transgene expression following exposure of tumor cells to hypoxic stress, we transfected pVEGF-UF5 into the SAU cell line and exposed these transfected cells to various lengths of hypoxic stress (Figure 2-7). No significant change in the number of GFP expressing cells was observed following exposure of the transfected cells to hypoxic conditions for up to 24 hours. Similar results were obtained using pVEGF-UF7. The plasmids, pEgr-1-UF5 and pTNF-UF5, were transfected into the SAU cell line and these transfected cells were exposed to various doses of ionizing radiation. A dose-dependent increase in the number of GFP expressing cells was observed 24 hours following radiation exposure of cells transfected with pEgr-1 (Figure 2-8). However, this increase occurred only at the highest clinically irrelevant dose of 20 Gy. No significant increase in the number of GFP expressing cells was observed following radiation exposure of cells transfected with pTNF-UF5 at the doses tested (Figure 2-9). Similar results were obtained following transfection of these cells with pEGR-1-UF7 and pTNF-UF7.

Chemotherapy Inducible Promoter: Gadd 153

The ability of a fragment of the Gadd 153 promoter to control transgene expression following exposure of tumor cells to various doses of the bioreductive agent MMC was tested in the SAU cell line. Exposure of these transfected cells to MMC for 24 hours resulted in an ~3-fold increase in cells scored positive for GFP expression (Figure 2-10). As with the radiation-inducible promoters, this increase occurred at clinically irrelevant doses. Similar results were obtained with pGadd153-UF7.

Expression of NQO1

The human ovarian tumor cell line, SAU, and the mouse sarcoma cell line, KHT/iv, were chosen for this study because they constitutively expressed very low levels of NQO1 protein. Liposome-mediated transfection of the proviral AAV vector plasmid, pTRUF5-NQO1, into these tumor cell lines significantly increased the total NQO1 protein in whole cell extracts (Figure 2-11a). For example, 24 hr after transfection the level of the ~30 kD NQO1 protein increased ~66-fold and ~102-fold in the SAU and KHT/iv cell lines, respectively.

To determine the effect on overall NQO1 activity cell sonicates were prepared from each cell line 24 hr following transfection with pTRUF5-NQO1 (Figure 2-11b). The activity in untransfected SAU cells could not be detected, but it increased to ~200 nmol/min/mg in cells transfected with the vector plasmid. In KHT/iv cells the activity was found to increase ~10-fold. Similar levels of NQO1 expression were observed following transfection with pTRUF7-NQO1.

Expression of the Green Fluorescent Protein

FACS analysis of SAU and KHT/iv cells following transfection with pTRUF5-NQO1 gave an estimation of transfection efficiency of these cell lines with this liposome-mediated transfection protocol (Figure 2-11c). Approximately 57% of SAU cells enter the M2 gate set on the basis of the untransfected SAU cells. The KHT/iv cell line's transfection efficiency with this protocol was somewhat lower with ~ 33% of the cells entering the M2 gate. Similar levels of GFP expression were observed following transfection with pTRUF7-NQO1.

Discussion

All of the stress-induced promoter fragments employed in these studies have been shown by other groups to result in increases in the relative expression of the luciferase or chloramphenical acetyl transferase (CAT) reporter genes. Inherent in such reporter assays is an amplification of the level of gene expression into a detectable range. The fluorescent signal from GFP is a significantly less sensitive reporter gene that was chosen for this work because of its ability to be detected visually and with FACS analysis. However, the transcriptional activation capacity of the various environmental stress induced promoter fragments appears to fall well below that required to adequately quantitate using this reporter gene. In addition, detectable GFP expression with these plasmids required the employment of doses that are clinically irrelevant. However, the use of this reporter raises an important issue for the development of transcriptionally targeted gene based approaches to solid tumors. In the development of such approaches it is important to consider not only the relative induction of the delivered transgene, but also the absolute amount of transgene expression. While the above promoter regions have been observed to increase gene expression following the appropriate stimuli relative to unstressed tumor cells using luciferase or CAT, the choice of GFP as a reporter indicates that the absolute levels of expression from these constructs appears to be quite low relative to a constitutively active promoter such as CMV. Further development of these approaches may require that multiple copies of these stress induced response elements are combined together or they may need to be combined with other minimal constitutively active promoters. While the transcriptional targeting approaches tested here still hold potential in their application to treating solid tumors, clearly further investigation into improving the relative selectivity as well as the absolute potency of these vectors are warranted.

On the other hand, the proviral plasmids, pTRUF7-NQO1 and pTRUF5-NQO1 had robust expression of the delivered transgenes following tranfection. NQO1 activity has been measured in many mammalian tissues and all measurements of NQO1 activity using menadione as a substrate in human tissues are less than 120 nmol/min/mg. Significantly higher levels of NQO1 expression are achievable 24 hours following transfection of these plasmids into both human and mouse tumor cell lines (Figure 2-11).

Indeed, the proviral AAV vector plasmid, pTRUF5-NQO1 (Fig. 2-6), demonstrated that the NQO1 gene and the reporter GFP could be expressed from a single dicistronic unit. Similar results also were observed with pTRUF7-NQO1. Figure 2-11a shows that both SAU and KHT/iv exhibited large increases in NQO1 protein level at 24 hr after transfection (66-fold and 102-fold, respectively). The overall NQO1 activity of these tumor cells also was increased significantly following transfection with pTRUF5-NQO1 (Figure 2-11b). Interestingly, compared to the human SAU cells, mouse KHT/iv cells showed a larger increase in NQO1 levels and a lower level of GFP expression (Figure 2-11a and c). One possible explanation for this observation may be that the CMV promoter is a stronger promoter in the KHT/iv line, while the IRES element is less efficient for translation of GFP in KHT/iv than in SAU. Still, the data at hand demonstrate that the NQO1 gene and the reporter GFP can both be expressed in mammalian tumor cell lines that are deficient in NQO1 protein.

In conclusion, while the stress responsive promoter constructs were ineffective at expressing GFP to a sufficiently detectable level, the proviral plasmids, pTRUF5-NQO1 and pTRUF7-NQO1 allow coordinate expression of the NQO1 gene and the reporter GFP from the same CMV promoter. Efficient transduction and overexpression of these genes in SAU and KHT/iv tumor cells, lines that are inherently deficient in NQO1 was observed. Following the production of recombinant AAV and adenoviral vectors from these plasmids, it will be possible to undertake VDEPT studies to determine whether such a treatment strategy can be utilized to enhance the antitumor efficacy of bioreductive anticancer agents that have been demonstrated to be substrates for NQO1.

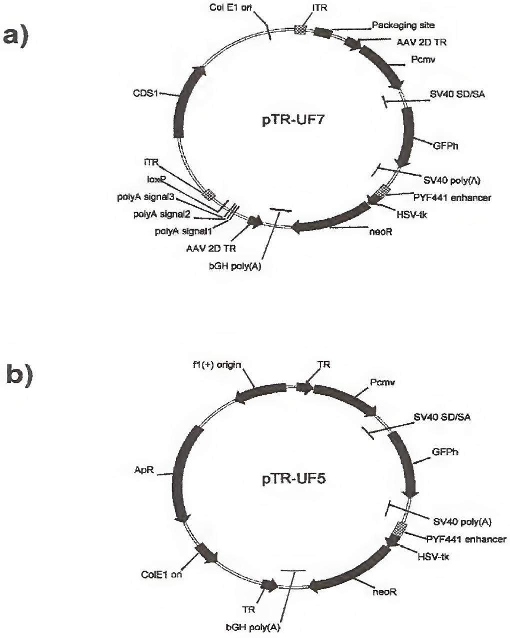


Figure 2-1. a) Adenovirus proviral vector plasmid, pTRUF7, containing an expression cassette with a CMV promoter controlling expression of GFP. b) AAV proviral plasmid, pTRUF5, containing the same expression cassette.

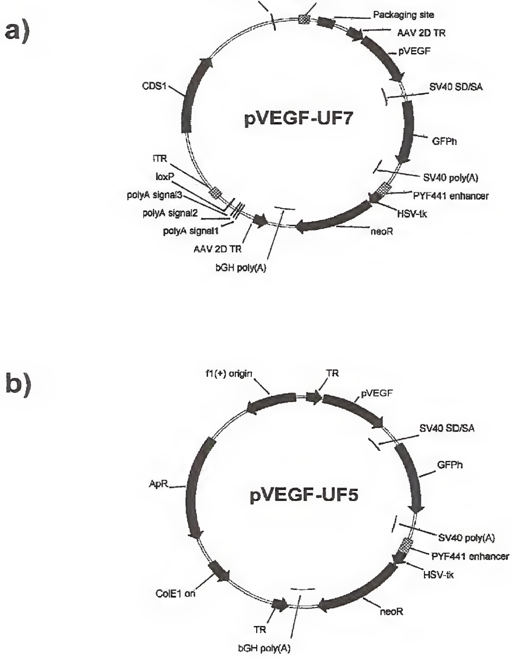


Figure 2-2. a) Adenovirus proviral vector plasmid, pVEGF-UF7, containing an expression cassette with a fragment of the human VEGF promoter controlling expression of GFP. b) AAV proviral vector plasmid, pVEGF-UF5, containing the same expression cassette.

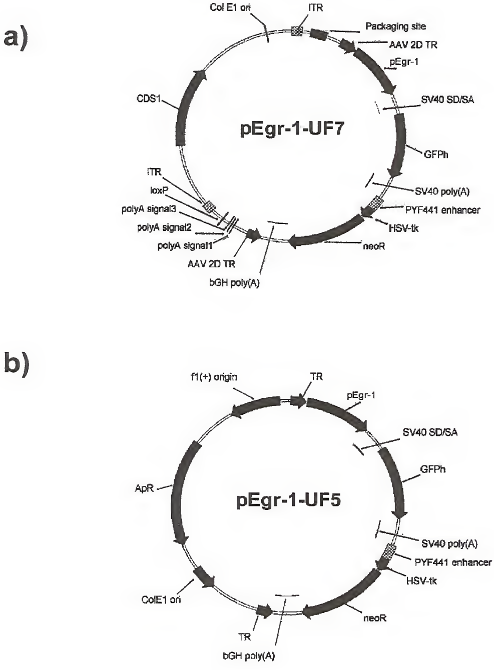


Figure 2-3. a) Adenovirus proviral vector plasmid, pEgr-1-UF7, containing an expression cassette with a fragment of the human Egr-1 promoter controlling expression of GFP. b) AAV proviral vector plasmid, pEgr-1-UF5, containing the same expression cassette.

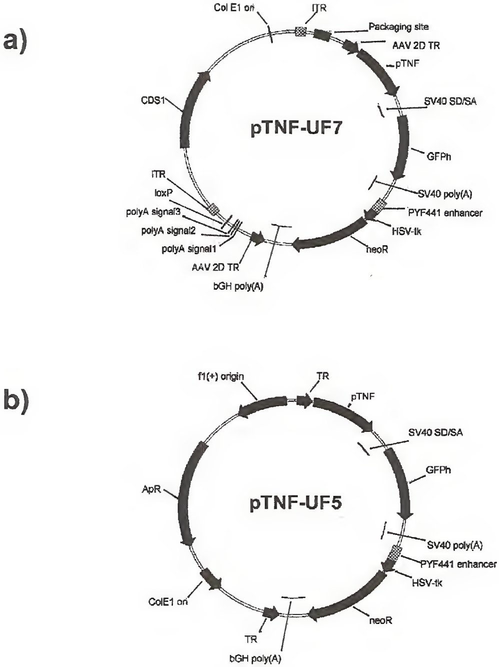


Figure 2-4. a) Adenovirus proviral vector plasmid, pTNF-UF7, containing an expression cassette with a fragment of the human TNF alpha promoter controlling expression of GFP. b) AAV proviral vector plasmid, pTNF-UF5, containing the same expression cassette.

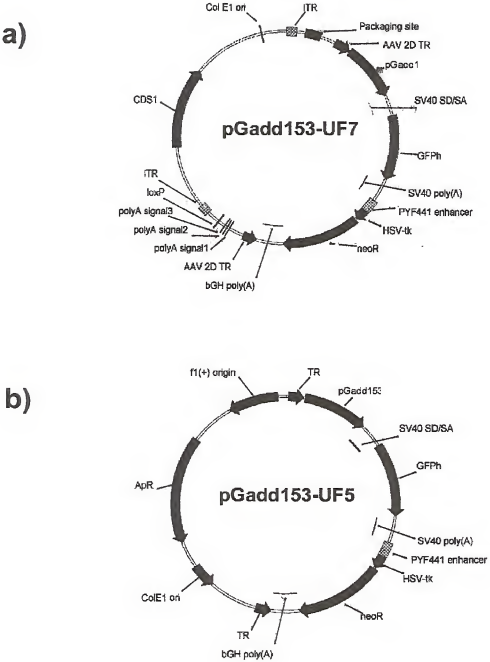


Figure 2-5. a) Adenovirus proviral vector plasmid, pGadd153-UF7, containing an expression cassette with a fragment of the human Gadd 153 promoter controlling expression of GFP. b) AAV proviral vector plasmid, pGadd153-UF5, containing the same expression cassette.

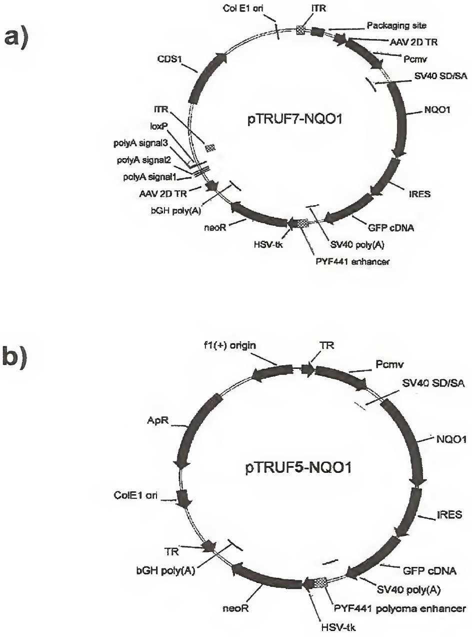


Figure 2-6. a) Adenovirus proviral vector plasmid, pTRUF7-NQO1, containing an expression cassette with a CMV promoter controlling expression of a dicistronic unit containing NQO1 and GFP.
b) AAV proviral vector plasmid, pTRUF5-NQO1, containing the same expression cassette.

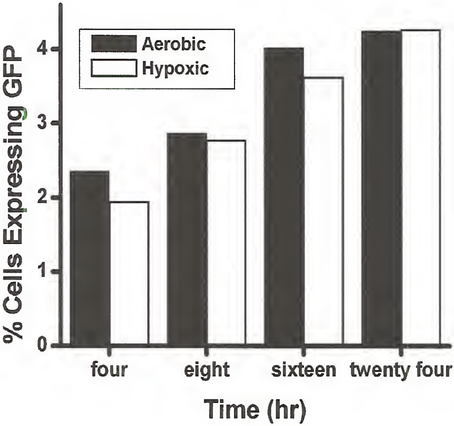


Figure 2-7. Analysis of GFP expression in SAU cells following transfection with pVEGF-UF5 and exposure to hypoxic conditions for various durations of time.

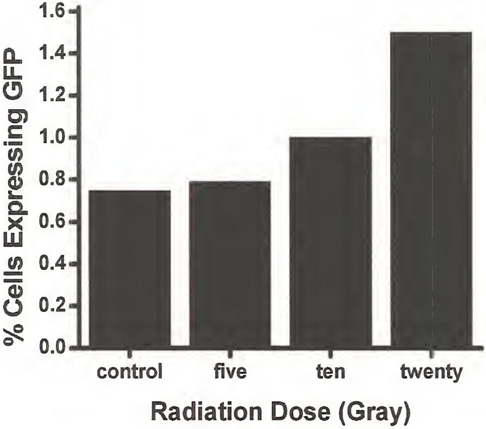


Figure 2-8. Analysis of GFP expression in SAU cells following transfection with pEgr1-UF5 24 hours after treatment with various doses of ionizing radiation.

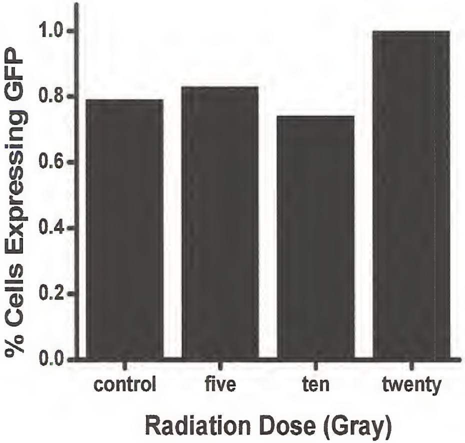


Figure 2-9. Analysis of GFP expression in SAU cells following transfection with pTNF-UF5 24 hours after treatment with various doses of ionizing radiation

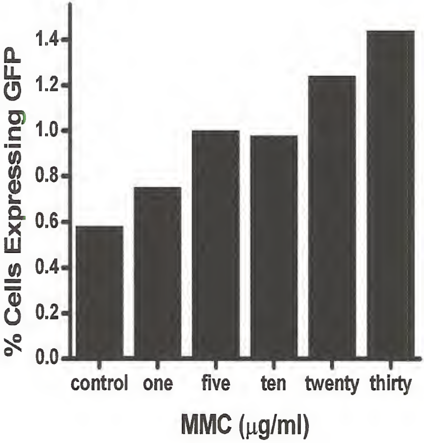


Figure 2-10. Analysis of GFP expression in SAU cells following transfection with pGadd153-UF5 24 hours after treatment with various doses of mitomycin C.

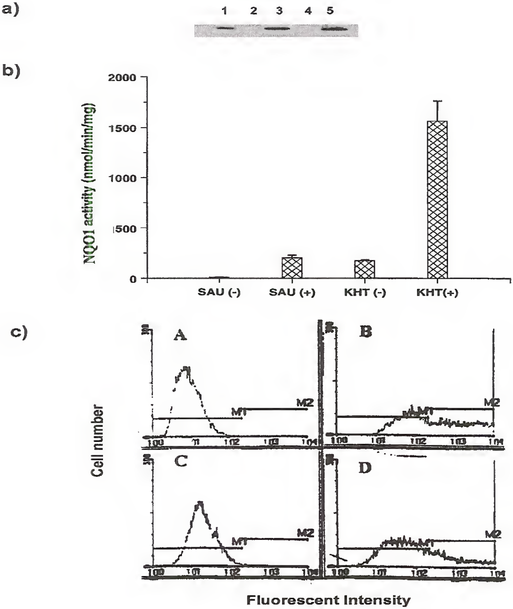


Figure 2-11. Analysis of pTRUF5-NQO1 protein expression. a) Western blot analysis for NQO1. Lane 1: NQO1 standard; Lane 2: SAU; Lane 3: SAU, 24 hours post transfection with pTRUF5-NQO1; Lane 4: KHT/iv; Lane 5: KHT/iv, 24 hours post transfection with pTRUF5-NQO1. b) Analysis of NQO1 activity before (-) and after (+) transfection with pTRUF5-NQO1. c) Analysis of GFP expression by FACS. A: SAU; B: SAU, 24 hours post transfection; C: KHT/iv; D: KHT/iv, 24 hours post transfection.

CHAPTER 3

ENZYME/DRUG RELATIONSHIPS FOR BIOREDUCTIVE CHEMOTHERAPY IN HUMAN OVARIAN CARCINOMA CELLS: EXPLOITATION OF GENE BASED ENZYME-DIRECTED TREATMENT STRATEGIES USING AN AAV PROVIRAL PLASMID

Introduction

The enzyme-directed strategy for bioreductive chemotherapy is designed to take advantage of two aspects of solid tumors in order to improve the therapeutic index (Workman, 1994). The selectivity of bioreductive chemotherapy has the potential to be governed not only by the oxygenation difference between tumors and normal tissue but also differences in the expression of the enzymes catalyzing the reductive metabolism of these agents. In this concept of enzyme directed bioreductive anticancer therapy, the choice of the bioreductive agent could be based on the presence of hypoxic tumor cells as well as the enzymatic profile of the tumor. Once particular enzyme/drug relationships have been identified, it may be possible to further enhance the intratumoral cytotoxicity of these agents through the genetic delivery of these reducing enzymes to the tumor and their subsequent overexpression prior to administration of the appropriate chemotherapeutic substrate. Overexpression of the enzyme may be effective in this approach if it is responsible for the aerobic and/or the hypoxic metabolic bioactivation. Indeed, this approach may be especially appropriate in tumors determined to be inherently low in these enzymes and/or harbor mutated forms of the enzyme rendering it nonfunctional (Traver *et al.*, 1992).

One approach to the delivery of these enzymes to the tumor cells is through the use of a recombinant viral vector. Adeno-associated virus (AAV) is a relatively new member of the current viral gene delivery systems (Muzyczka, 1992). One advantage of this type of vector is that AAV is a defective parvovirus that requires coinfection by a second unrelated helper virus, such as adenovirus or herpes virus, for productive infection. In the absence of helper virus coinfection, AAV remains latent within the cell by integrating into the host genome. The viral genome contains two open reading frames: the *rep* gene, which encodes the proteins necessary for viral replication, and the *cap* gene for the structural capsid proteins. These genes are flanked by two short inverted terminal repeats which are the only genomic elements required *in cis* for replication, integration, and packaging. To create an AAV vector, the *rep* and *cap* genes are removed from a proviral plasmid and replaced by the desired DNA fragment to generate an AAV vector plasmid. This proviral plasmid can then be used to test the expression of the desired gene within an AAV background. Recombinant AAV viral vectors can subsequently be produced by transfecting this AAV vector plasmid into a suitable tissue culture cell (e.g., 293 cells), and supplying *rep*, *cap*, and the appropriate helper functions *in trans*.

Ovarian carcinoma remains the leading cause of death of women due to gynecological malignancies in several industrialized nations. Due to the lack of effective screening strategies along with the relative lack of symptoms with early stage disease, most women present with advanced stage ovarian cancer. This advanced stage disease is often refractory to conventional anticancer treatment modalities. Therefore, new treatment strategies for ovarian cancer need to be investigated. The large size of ovarian carcinomas may result in extensive areas of the tumor that are poorly vascularized and, thus, oxygen deprived. The presence of these large hypoxic fractions in ovarian tumors also makes them potential candidates for bioreductive chemotherapy. Furthermore, knowledge of the expression of the bioreductive enzymes in ovarian carcinoma may allow for the employment of these bioreductive anticancer agents in an enzyme-directed approach.

In order to define enzyme/ bioreductive drug relationships for ovarian carcinoma, we examined the relationship between oxygenation status, enzyme profile, and the cytotoxicity of mitomycin C (MMC), EO9, and tirapazamine in a panel of 6 human ovarian tumor cell lines established from patient biopsies. The enzymes assessed in detail were DT-diaphorase (NQO1) and cytochrome P450 reductase (CYPOR). Based on the observed relationship between NQO1 activity and EO9 aerobic cytotoxicity in these cell lines, subsequent experiments were performed to investigate whether overexpression of NQO1 in 2 tumor lines deficient in NQO1 could sensitize them to aerobic treatment with EO9.

Materials and Methods

Cell Lines and Reagents

The human ovarian adenocarcinoma cell lines MLS, PEA, GRA, SKA, and SAU were established at the University of Rochester, Rochester, NY directly from patient biopsies (Lee *et al.*, 1989). OW-1 was received from Dr. R. Buick, Princess Margaret Hospital, Toronto, Canada. All cell lines were grown in DMEM supplemented with 10% FCS. EO9 was a gift from Professor GE Adams, MRC Laboratories, Harwell, UK. MMC was obtained from Bristol Laboratories. Tirapazamine was a gift from Dr. M. Tracy, SRI, Palo Alto, California. The NQO1 cDNA and anti-NQO1 antibody were a gift from Dr. D. Ross, Colorado State University.

Clonogenic Cell Survival Assay

Drug activities were determined under aerobic (95% air: 5% CO2) or hypoxic (95% N2: 5% CO2) conditions using *in vitro* clonogenic survival assays. Cells were harvested by trypsinization during exponential growth phase, then incubated in complete DMEM at 37° C in a spinner system utilizing Type 1 vials. The medium was gassed for 3 hr with either gas mixture before addition of cells, and throughout the experiment. Cells were exposed to MMC for 1 hr, EO9 for 3 hr, and tirapazamine for 4 hr. At the end of the treatment period, cells were collected by centrifugation and resuspended in fresh medium. Viable cell counts were determined, and the cells were plated into 60 mm plastic dishes containing DMEM. After 10-14 days the plates were stained with crystal violet and colonies containing more than 50 colonies were counted.

Reductase Enzyme Assay

NQO1 and CYPOR activities were determined in sonicates prepared from exponentially dividing cells. After trypsinization and washing with ice-cold phosphate buffered saline, cells were resuspended in ice-cold 0.25 M sucrose at a concentration of 10^7 cells/ml and sonicated on ice for 25 sec. Sonicates were stored at –80° C until assay. Protein content of sonicates was determined by the Bradford method. NQO1 activity was determined spectrophotometrically at 550 nm. Reactions were run at 37° C in a total volume of 1 ml in 25 mM TRIS-HCl buffer, pH 7.4, containing 77μM cytochrome c, 20 μM menadione, 2 mM NADPH, and 0.14% BSA. The assay was initiated by addition of the sample to the reaction mixture. NQO1 activity was calculated (using an extinction coefficient of $21.1\text{x}10^{-3}$ $\text{mol}^{-1}\text{Lcm}^{-1}$) as the difference between total enzyme activity and that inhibited by the addition of 100 μM dicoumarol to the reaction mixture. CYPOR activity was determined at 37° C by a spectrophotometric assay. The 1 ml reaction mixture consisted of 100 mM potassium phosphate buffer, pH 7.4, 50 μM cytochrome c, 1 mM KCN, and 100 μM NADPH. The reaction was initiated by addition of NADPH; control samples were run in the absence of NADPH. Enzyme activity was measured by following cytochrome c reduction at 550 nm (using a molar extinction coefficient of ($21.1\text{x}10^{-3}\text{mol}^{-1}\text{Lcm}^{-1}$).

Plasmid Construction, Transfection, and Drug Treatment

The plasmids were transfected into the SKA and SAU cell lines by liposome mediated transfection with Lipofectamine Plus (GIBCO/BRL) using the manufacturer's guidelines. Briefly, cells were plated for 24 hr in 6-well dishes until they reached 50-70% confluence. The liposome-DNA complexes then were added to the cells in serum-free medium and incubated for 5 hr at 37 °C. This mixture was removed and replaced with fresh complete medium, and the cells were incubated at 37°C for an additional 72 hr. At this time the cells were plated in medium containing 1 mg/ml G418 and cultured for 6-8 weeks. Individual clones were isolated, trypsinized, and clonally expanded. SKA/N and SAU/N clones, overexpressing NQO1, were treated as exponentially growing monolayers with EO9 at 37° C under aerobic conditions for 3 hours. At the end of the treatment period, cells were trypsinized, collected by centrifugation, and resuspended in fresh medium. Viable cell counts were determined, and the cells were plated into 60 mm plastic dishes containing DMEM. After 10-14 days the plates were stained with crystal violet and colonies containing more than 50 colonies were counted.

Preparation of Whole Cell Extracts and Western Blotting

Extracts were made from clonal populations of SKA/N and SAU/N cells. Extracts were prepared using an extraction buffer containing 50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% NP40 (v/v), 1 mM PMSF, aprotinin, and leupeptin (EBC buffer). Cells were washed with cold PBS, resuspended in EBC buffer, incubated at room temperature on a rocking platform for 10 min, centrifuged at 1100 rpm for 10 min, and the supernatant was saved. Protein determinations (for gel loading) were made on the extracts by Bradford Assay (BIO-RAD). Fifty μg of total cellular protein was subject to SDS-PAGE (12% polyacrylamide). Following gel run, the proteins were transferred from the gel to nitrocellulose membranes at 300 mA at 4°C for 3 hr. The membranes were washed with Tris-buffered saline (TBS) and blocked overnight with 5% dry milk in TBS solution at 4°C. The membranes were then incubated for 1 hr at room temperature with 5 ml of hybridoma supernatant containing an anti-NQO1 monoclonal antibody. The membranes were then washed with TBS for 30 min, and incubated with a 1:5000 dilution of a goat anti-mouse IgG conjugated to HRP (Promega, Madison, WI) for 30 min at room temperature. The blots were developed using chemiluminescence and the quantity of protein was determined using densitometry.

Results

Oxygenation Status and Cytotoxicity

The efficacy of the bioreductive agents EO9, MMC, and tirapazamine on the survival of the MLS ovarian tumor cells under aerobic and hypoxic conditions is illustrated in Figure 3-1. Similar analyses were performed for all six human ovarian tumor cell lines and the IC_{90} values (doses that kill 90% of cells) and aerobic/hypoxic differentials (aerobic IC_{90}/hypoxic IC_{90}) are presented in Table 3-1. All three agents were found to be active under both aerobic and hypoxic conditions, though they differed considerably in their selectivity for hypoxic cells. EO9 was the most potent of the three drugs tested. Cells treated under hypoxic conditions exhibited comparable IC_{90} values for all cell lines (0.01 to 0.075 μM). Cells treated under aerobic conditions showed a more variable response with IC_{90} values ranging from 0.1 to 4.4 μM. As a consequence the resultant aerobic/hypoxic differentials for EO9 varied from ~3-60. Cells treated with the quinone antibiotic MMC were found to be less sensitive to this agent than to EO9, and in contrast to EO9, showed no aerobic/hypoxic differential. As has been shown previously (Skarsgard *et al.*, 1994), the N-oxide tirapazamine possessed a high degree of selective cytotoxicity for hypoxic cells. In the ovarian tumor cell lines, treatment with tirapazamine led to aerobic/hypoxic differentials ranging from ~40-135.

Enzyme Levels and Cytotoxicity

The activities of 2 cellular reductases, NQO1 and CYPOR were examined in this panel of ovarian cell lines. Marked differences in NQO1 activity were observed (Fig 3-2a) with an ~150-fold difference between the highest and lowest cell lines (MLS and SAU, respectively). In contrast, all six lines demonstrated similar levels of CYPOR (Fig 3-2b). To assess the relationship between enzyme activity and drug efficacy, plots relating these parameters under the various treatment conditions for all three agents were constructed. For EO9 this evaluation revealed a strong correlation between NQO1 enzyme level and drug sensitivity under aerobic conditions (Figure 3-3a). The results clearly illustrated that cell lines with the higher NQO1 activity exhibited greater cytotoxicity in response to EO9 than did cell lines with lower

NQO1 activity. For example, the MLS cell line had the highest NQO1 activity and the lowest aerobic IC_{90}, and the opposite was true for the SAU cell line. Analysis of the results illustrated in Table 3-1 and Figure 3-2 further showed no correlation between IC_{90} and (i) CYPOR activity for EO9, and (ii) NQO1 activity for tirapazamine. For tirapazamine the data are suggestive of a correlation between hypoxic drug efficacy and the activity of CYPOR in these ovarian tumor cells (Figure 3-3b), as the cell lines exhibit overlapping enzyme activities as well as IC_{90} doses. However, CYPOR values vary by a factor of only 1-3 in these cell lines, therefore conclusions concerning their impact on tirapazamine activity can not be ascertained. Finally, this evaluation revealed no correlation between enzyme activities and drug efficacy for MMC in these cell lines.

To further examine the NQO1/EO9 relationship, MLS and SAU cells were exposed to the NQO1 inhibitor dicoumarol prior to treatment with EO9. The results showed that inhibition of NQO1 in the MLS cell line by dicoumarol resulted in a marked decrease in toxicity under aerobic, but not hypoxic, conditions (Figure 3-4a). In contrast, pretreatment of the SAU cell line with dicoumarol under either aerobic or hypoxic conditions had no effect on the cytotoxicity of EO9 (Figure 3-4b).

NQO1 Stable Transfectants and EO9 Cytotoxicity

To more directly demonstrate the role of the reducing enzyme NQO1 in the aerobic cytotoxicity of EO9, transfection experiments to increase the level/activity of this enzyme in cell lines that were inherently low were undertaken. MLS, SKA, and SAU cell lines were examined. The MLS cell line was chosen for its high level of NQO1 activity while the SKA and SAU cell lines possessed the lowest NQO1 activities of all the lines tested. As expected from the NQO1 activity measurements (Figure 3-2a), the MLS line had the highest amount of NQO1 protein, whereas the SKA and SAU lines had much lower levels (Figure 3-5).

To determine if increasing the level of NQO1 expression in cell lines inherently low in this enzyme would increase their sensitivity to EO9, the SKA and SAU cell lines were stably transfected with the AAV vector plasmid pTRUF5-NQO1. SKA/N and SAU/N clones of SKA and SAU that were overexpressing NQO1 protein to a similar level (Figure 3-6a and b) were selected. The NQO1 activity of SKA/N and SAU/N also were determined and found to be similar to each other (Figure 3-6c). The efficacy of EO9 in transfected and parental SAU and SKA tumor cells treated under aerobic conditions is illustrated in 7. A comparison of IC_{90} values shows that the transfected cells are ~3-fold more sensitive to this chemotherapeutic agent than the parental cell lines, and this increase was inhibited by pretreatment with dicoumarol.

Discussion

In the present investigation we observed that EO9, MMC, and tirapazamine were effective at killing human ovarian tumor cells. In these 6 ovarian tumor cell lines MMC revealed only a modest aerobic/hypoxic differential (Figure 3-1, Table 3-1). The importance of this parameter in MMC toxicity to tumor cells has been a matter of controversy. MMC has been reported to be preferentially toxic to hypoxic cells *in vitro* and *in vivo* in some studies, but not in others (Skarsgard *et al.*, 1994; Mikami *et al.*, 1996). Further, while our results suggest that NQO1 and CYPOR may not be important for MMC toxicity in ovarian tumor cells (Table 3-1, Figure 3-2), others have reported a correlation between these reductases and sensitivity to MMC in other tumor models. For example, MMC sensitivity and NQO1 gene expression were examined in a series of colon carcinoma cell lines and it was observed that MMC sensitivity was related to NQO1 gene expression, with high expression of NQO1 resulting in increased sensitivity to MMC in these cell lines (Traver *et al.*, 1992). Others have suggested that the correlation between cellular NQO1 activity and MMC sensitivity is questionable (Robertson *et al.*, 1992), and may be more dependent on the environmental pH during treatment (Siegel *et al.*, 1993). It is possible that the role of NQO1 in MMC toxicity is tissue-specific and determined, in part, by the activities of other cellular reductases. However, because no clear relationship between MMC and NQO1 or CYPOR was observed in our panel of ovarian cell lines, these enzyme/drug combinations were not pursued further in our gene-based approach.

Unlike MMC, and as has been observed by others (Skarsgard *et al.*, 1994), the cytotoxic effects of tirapazamine exhibited a strong dependence on the oxygenation status during treatment. Similar to MMC, tirapazamine demonstrated no correlation between the level of NQO1 activity and the response of the panel of ovarian tumor cell lines to treatment. Also, because the six ovarian cell lines exhibited overlapping CYPOR activities, no relationship could be established between this enzyme and the cytotoxicity of tirapazamine (Figure 3-2b). The narrow range of IC_{90} values observed for tirapazamine in these lines suggests that this may be due to low variation in CYPOR activity. While the importance of this reductase in the bioactivation and cytotoxicity of tirapazamine in these ovarian tumor cells remains unequivocal, it should be noted that others have reported the aerobic and hypoxic cytotoxicity of tirapazamine toward human breast tumor lines to be correlated with CYPOR expression (Patterson *et al.*, 1997; Patterson *et al.*, 1995). The present experiments also failed to support the notion that NQO1 expression in tumor cells leads to a reduction in the efficacy of tirapazamine as has been noted previously (Patterson *et al.*, 1994). This suggests that other tissue specific characteristics may be involved in the detoxification of tirapazamine by NQO1.

In contrast to MMC and tirapazamine, we observed that both cellular oxygenation status and NQO1 activity to be important determinants of EO9 cytotoxicity. While EO9 demonstrated significant hypoxia selective cytotoxicity, NQO1 activity correlated with EO9 cytotoxicity under aerobic conditions (Figure 3-3a). The ovarian cell lines with higher NQO1 activity exhibited greater cytotoxicity in response to EO9 treatment. No such correlation was observed under hypoxic conditions (Table 3-1). The level of NQO1 activity in these ovarian cell lines was determined to be the result of the amount of NQO1 protein present in cell extracts. The inhibition of NQO1 with dicoumarol prior to treatment with EO9 in the high expressing MLS cell line resulted in a marked decrease in toxicity under aerobic, but not hypoxic, conditions (Figure 3-4a). Pretreatment with dicoumarol in the lowest NQO1 expressing cell line, SAU, had no effect on drug efficacy (Figure 3-4b). These results are in agreement with several studies of EO9 cytotoxicity in other tumor tissue types. A strong correlation was revealed between cellular NQO1 activity and response to E09 under aerobic conditions in a panel of 15 human tumor cell lines, including breast, lung, and colon carcinoma lines (Robertson *et al.*, 1994). In addition, similar results were observed in a study of EO9 chemosensitivity and NQO1 gene expression in a panel of seven human and four murine tumor cell lines (Smitskamp-Wilms *et al.*, 1995). Moreover, overexpression of NQO1 in CHO cells has been shown to result in an increase in the aerobic sensitivity of these cells to such agents as EO9, streptonigrin, and diaziquone (Gustafson *et al.*, 1996). Finally, induction of NQO1 by the pretreatment of L5178Y murine lymphoma cells with the 1,2-dithiole-3-thione, oltipraz, led to an increase in the aerobic toxicity of EO9 (Begleiter *et al.*, 1996).

Taken together, these results provide evidence that the NQO1/EO9 combination has the potential to be exploited in an enzyme-directed approach to bioreductive chemotherapy. To further test this possibility, the two lines expressing the lowest levels of NQO1, SKA and SAU (Figure 3-5) were stably transfected with an AAV proviral vector plasmid containing the cDNA for

NQO1 under control of the CMV promoter. The clones, SKA/N and SAU/N were chosen for their similar levels of increased NQO1 expression (Figure 3-6) and treated with EO9 under aerobic conditions (Figure 3-7). The similar increases in response to treatment with EO9 under aerobic conditions in these clones, and the ability to inhibit this increase with pretreatment with dicoumarol, provides further evidence for the strong association between this enzyme/drug combination. Furthermore, NQO1 can be effectively expressed in an adenovirus and an AAV background. These observations have set the stage for the production of recombinant viral vectors expressing the NQO1 gene for use *in vitro* and *in vivo* in virally directed enzyme prodrug therapies with bioreductive substrates for NQO1 such as EO9.

In summary, ovarian carcinoma appears to have clinical characteristics that make it a promising choice for gene therapy treatment strategies. Most patients have disease contained within the peritoneal cavity at the time of diagnosis. This localization of the disease would allow for the delivery of recombinant viral vectors to the cavity through intraperitoneal injection. By facilitating effective *in vivo* gene transfer, the confinement of these tumors within the peritoneal cavity might also prove to be an advantageous safety factor, decreasing the potential for genetic transfer to normal tissues.

In the present investigations, an examination of the relationship between oxygenation status, enzyme profile, and the cytotoxicity of three bioreductive agents in a panel of human ovarian tumor cell lines revealed a strong relationship between the activity of the enzyme, NQO1, and the efficacy of the quinone, EO9. While EO9 is a potent hypoxic cytotoxin in this panel of ovarian tumor cell lines, it appears that this NQO1/EO9 relationship has the potential to be exploited under aerobic conditions in an enzyme-directed approach to improve the overall cytotoxicity of this agent. Furthermore, it is also possible to improve the aerobic killing effects of EO9 through the genetic delivery and subsequent overexpression of NQO1 to ovarian tumor cells inherently low in this enzyme. These experiments have set the stage for examining the ability of improving the response of low NQO1 expressing human ovarian tumor xenografts to EO9 *in vivo* by delivering and overexpressing NQO1, using a viral vector such as adeno-associated virus, prior to administration of this bioreductive chemotherapeutic agent.

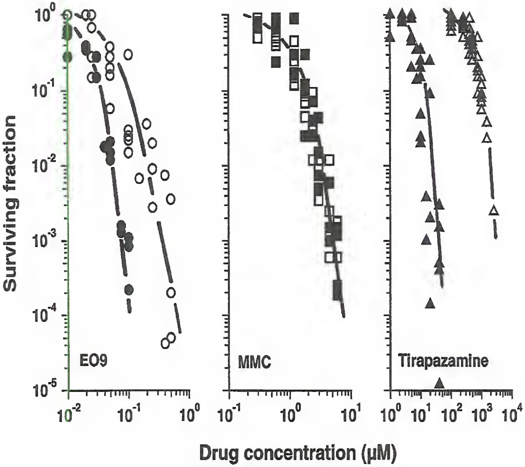


Figure 3-1. The effects of three bioreductive chemotherapy agents (EO9, MMC, tirapazamine) on the survival of MLS ovarian tumor cells treated under aerobic (open symbols) and hypoxic (closed symbols) conditions. Survival is expressed as a ratio of plating efficiencies of treated and control cells.

	EO9			Mitomycin C			Tirapazamine		
Cell Line	Air	N_2	Air/N_2	Air	N_2	Air/N_2	Air	N_2	Air/N_2
MLS	0.1	0.03	3.3	1.8	1.8	1.0	970	13	75
OW1	0.3	0.01	30	2.1	2.1	1.0	630	14	45
PEA	0.7	0.03	23	1.7	1.7	1.0	1080	8	135
GRA	1.6	0.05	32	6.0	3.9	1.5	1035	15	69
SKA	1.8	0.045	40	2.4	1.8	1.3	780	12	65
SAU	4.4	0.075	59	5.4	3.9	1.4	440	11	40

Table 3-1. IC_{90} values (μM) and aerobic/hypoxic differentials of three bioreductive chemotherapy agents (EO9, MMC, tirapazamine) for a panel of human ovarian tumor cell lines treated under aerobic and hypoxic conditions.

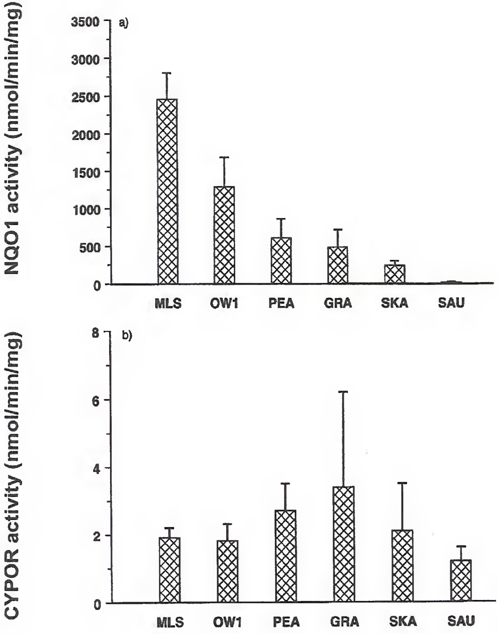


Figure 3-2. Analysis of the activities of NQO1 (a) and CYPOR (b) in a panel of human ovarian tumor cell lines.

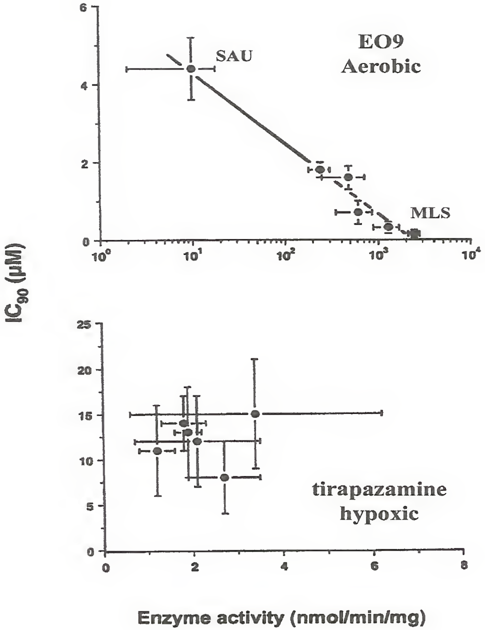


Figure 3-3. Relationship between IC_{90} dose and enzyme activity for EO9 and tirapazamine in a panel of human ovarian tumor cell lines. (a) NQO1/EO9, aerobic conditions; (b) CYPOR/tirapazamine, hypoxic conditions.

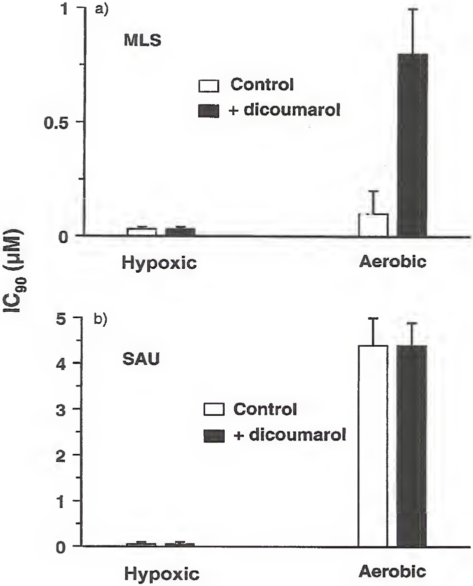


Figure 3-4. Effects of pretreatment of MLS (a) and SAU (b) cell lines with the NQO1 inhibitor, dicoumarol, prior to administration of EO9 under aerobic and hypoxic conditions.

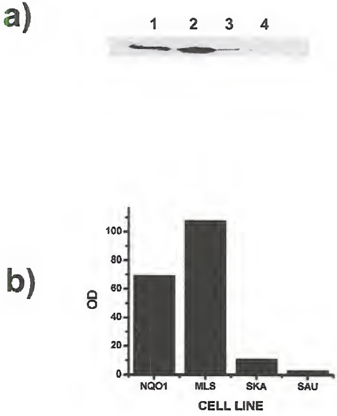


Figure 3-5. Analysis of NQO1 expression in the MLS, SKA, and SAU ovarian tumor cell lines by western blotting. a) Lane 1: NQO1 standard; Lane 2: MLS; Lane 3: SKA; Lane 4: SAU. b) Densitometric scan of gel in panel a).

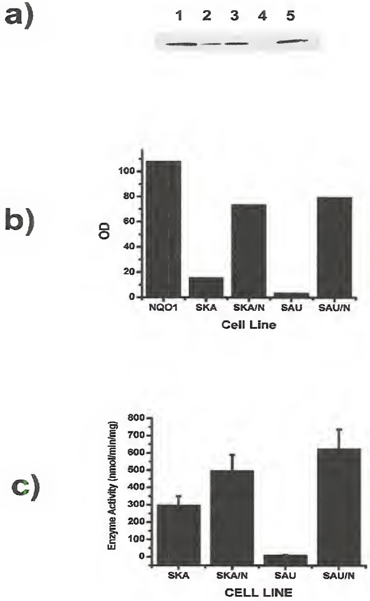


Figure 3-6. Analysis of NQO1 protein and activity levels in SKA, SKA/N, SAU, SAU/N cell lines. a) Western blot of NQO1 parental (SKA, SAU) and NQO1 overexpressing stable transfectants (SKA/N, SAU/N). Lane 1: NQO1 standard; Lane 2: SKA; Lane 3: SKA/N; Lane 4: SAU; Lane 5: SAU/N. b) Densitometric scan of gel in panel a). c) NQO1 activities in these cell lines.

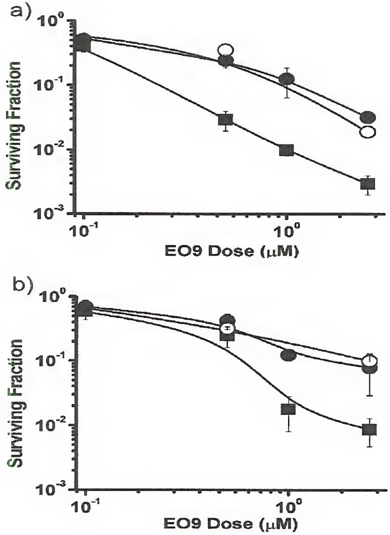


Figure 3-7. Effects of stable overexpression of NQO1 in SKA (a) and SAU (b) cells on sensitivity to EO9 treatment under aerobic conditions. Closed circles: SKA and SAU parental cells; Closed squares: SKA/N and SAU/N cells; Open circles: SKA/N and SAU/N cells pretreated with NQO1 inhibitor, dicoumarol, prior to administration of EO9.

CHAPTER 4

COMPARISON OF RECOMBINANT ADENO- AND ADENO-ASSOCIATED VIRAL GENE DELIVERY SYSTEMS FOR EMPLOYMENT IN A BIOREDUCTIVE ENZYME-DIRECTED PRODRUG STRATEGY

Introduction

The development of cancer gene therapy may provide opportunities for more tumor selective targeting of the toxic effects of chemotherapeutic agents. Virally directed enzyme/prodrug therapy (VDEPT) is one such approach currently under active investigation (Singhal and Kaiser, 1998). The underlying principle of this approach is to employ a recombinant viral vector to deliver a gene encoding an enzyme that transforms a nontoxic (or less toxic) prodrug into a toxic compound. Thus, the cells bearing the "suicide gene" are killed when the prodrug is administered. VDEPT, like conventional chemotherapy, relies on the delivery of a toxin to achieve tumor cell killing. However, the fundamental goal of this approach is to preferentially increase only the tumor cell's exposure to cytotoxic metabolites generated locally. Consequently, the therapeutic index would be improved due to more effective killing of infected tumor cells without an increase in normal tissue toxicity.

The herpes simplex virus thymidine kinase/ganciclovir system (Moolten, 1986) and the *E. coli* cytosine deaminase gene/5-fluorocytosine system are examples of such two-stage treatment strategies

(Huber, *et al.*, 1993). Both of these approaches employ an enzyme of non-mammalian origin. As a result, their effectiveness is entirely dependent on previously infected tumor cells encountering the prodrug. While this strategy has been observed to exhibit a bystander effect following drug activation (Touraine *et al.*, 1998), a disadvantage of the non-mammalian enzyme approach is that, in general, uninfected tumor cells may take up available prodrug, but remain totally unaffected by its cytotoxicity. An alternative VDEPT approach is the use of mammalian enzyme/prodrug combinations. An example is the cytochrome P450 2B1 gene/cyclophosphamide system (Wei *et al.*, 1994) which aims to improve upon the effectiveness of a clinically active anticancer agent. One advantage of employing a mammalian enzyme in a VDEPT strategy is that by overexpressing an enzyme that may already be present within the tumor at some level, it is not essential to the overall success of the therapy that the anticancer agent encounters a tumor cell that has previously been infected with the recombinant viral vector. Indeed, in the absence of bioactivating enzyme overexpression, the anticancer agent will still be cytotoxic to uninfected tumor cells. However, if the mammalian enzyme is delivered in order to replace a mutant, nonfunctional one, then this approach becomes similar to VDEPT approaches employing non-mammalian enzymes.

Current bioreductive anticancer agents may have the potential to be included in this type of VDEPT approach to killing tumor cells. Designed to preferentially kill hypoxic tumor cells, the quinone bioreductive agent, EO9, is a potent hypoxic cytotoxin in our panel of human ovarian tumor cell lines. In these tumor cells EO9 is less potent under aerobic conditions, yet this aerobic potency appears to be governed by the level of expression of the obligate 2-electron reductase, NQO1. Although enzymatic reduction of EO9 by NQO1 does not appear to be critical for hypoxic cell killing, this enzyme/drug relationship does have the potential to be exploited in a VDEPT approach to improving the overall tumor response to EO9 by enhancing the aerobic metabolism of this quinone. Indeed, those ovarian tumors that are inherently low in NQO1 expression or harbor a mutated nonfunctional NQO1 gene may benefit from the delivery of a recombinant viral vector expressing NQO1 prior to administration of EO9.

The vector is critical for gene delivery and expression of the transgene, but existing vectors all have limitations. Gene delivery systems that have shown promise for the *in vitro* and *in vivo* transfer of genetic material are the recombinant viral vectors. Currently, several different viral delivery systems are being investigated for use as vectors for cancer gene therapy including retroviruses, adenoviruses, herpes-simplex viruses, adeno-associated viruses, and hybrid viruses that contain salient features of two or more of these viruses (Robbins and Ghivizzani, 1998).

The purpose of this study was to investigate the ability of recombinant adenoviral and AAV vectors to deliver genes to SKA and SAU ovarian tumor cell lines. We examined the ability of recombinant viruses expressing GFP to infect and become transcriptionally active in these cell lines using fluorescent microscopy and FACS analysis. These NQO1-deficient cell lines were then infected with recombinant viruses that express NQO1 and GFP from a single promoter and gene expression was examined using fluorescent microscopy, FACS analysis, and western blotting. Finally, SKA and SAU cells infected with the NQO1 expressing recombinant viruses were treated with EO9 in a VDEPT approach to sensitizing them to its aerobic killing effects.

Materials and Methods

Proviral Plasmid Construction and Recombinant Virus Production

The adenovirus proviral plasmid, pTRUF7, contains a CMV promoter controlling expression of GFP, a single lox P site for use in *Cre*-recombinase production of recombinant virus, the neomycin resistance gene under control of a fragment of the herpes simplex thymidine kinase promoter, and the entire expression cassette is flanked by adenovirus ITRs (Figure 4-1a). The AAV proviral plasmid, pTRUF5, contains a CMV promoter controlling expression of GFP, the neomycin resistance gene under control of a fragment of the herpes simplex thymidine kinase promoter, and the AAV TRs flanks the entire expression cassette (Figure 4-1b). The adenoviral proviral plasmid, pTRUF7-NQO1, and the AAV proviral plasmid, pTRUF5-NQO1 were constructed by inserting a dicistronic unit containing the human NQO1 cDNA and GFP cDNA separated by a poliovirus type 1 IRES element downstream of the CMV promoter (Figure 4-2). Recombinant adenoviral vectors were produced using Cre-lox recombination (Hardy *et al.*, 1997). Recombinant AAV vectors were produced by the method of Grim *et al.* (1998). Recombinant AAV vectors were purified by the method of Zolotukhin et al. (1999).

Recombinant Virus Infection

The human ovarian tumor cell lines SKA and SAU were initially derived from patient biopsies and have been maintained as previously reported (Lee *et al*., 1989). For experiments, exponentially growing cell cultures were incubated in suspension with recombinant viral vectors at various MOI for 3 hours at 37° C in serum-free DMEM medium in a total volume not exceeding 100 μl. Following this incubation, the cell/viral suspension was plated in 60 mm dishes in complete DMEM and incubated at 37° C.

Fluorescent Microscopy and FACS Analysis

Following infection, SKA and SAU cells were visualized with fluorescent microscopy and photographed. The cells then were fixed in ice cold p-formaldehyde for 24 hours and analyzed by FACS (Becton Dickinson flow cytometer, University of Florida Core Facility for Flow Cytometry) to determine the percentage of tumor cells expressing GFP. Dead cells and debris were excluded from the analysis based on forward angle and side scatter light gating. A gate was set on the uninfected cell samples and cells whose fluorescence increased due to GFP expression were scored as positive if their fluorescence increase shifted them into a second gate.

Preparation of Whole Cell Extracts and Western Blotting

Following infection of SKA and SAU cells, extracts were prepared using an extraction buffer containing 50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% NP40 (v/v), 1 mM PMSF, aprotinin, and leupeptin (EBC buffer). Cells were washed with cold PBS, resuspended in EBC buffer, incubated at room temperature on a rocking platform for 10 min, centrifuged at 1100 rpm for 10 min, and the supernatant was saved. Protein determinations (for gel loading) were made on the extracts by Bradford Assay (BIO-RAD). Fifty μg of total cellular protein were subject to SDS-PAGE (12% polyacrylamide). Following gel run, the proteins were transferred from the gel to nitrocellulose membranes at 300 mA at 4°C for 3 hr. The membranes were washed with Tris-buffered saline (TBS) and blocked overnight with 5% dry milk in TBS solution at 4°C. The membranes were then incubated for 1 hr at room temperature with 5 ml of hybridoma supernatant containing an anti-NQO1 monoclonal antibody. The membranes were then washed with TBS for 30 min, and incubated with a 1:5000 dilution of a goat anti-mouse IgG conjugated to HRP (Promega, Madison, WI) for 30 min at room temperature. The blots were developed using chemiluminescence and the quantity of protein was determined using densitometry.

VDEPT Clonogenic Cell Survival Assay

Tumor cells were infected with the recombinant viral vectors in suspension and plated in 60 mm dishes for 24 hours. These exponentially growing monolayers of infected tumor cells then were treated with EO9 (a gift from Prof. G Adams) at 37° C under aerobic conditions for 3 hours. Following drug treatment, cells were trypsinized, collected by centrifugation, washed, and resuspended in fresh medium. Viable cell counts were determined by hemocytometer, and the cells were plated into 60 mm plastic dishes containing completed DMEM. After 10-14 days the plates were stained with crystal violet and colonies containing more than 50 colonies were counted.

Results

Infection with Recombinant Adeno and AAV expressing GFP

The ability of recombinant adenovirus, rUF7, and recombinant AAV, rUF5, to infect and express the reporter gene GFP was examined in the SKA and SAU ovarian tumor cell lines. Following infection with rUF7, GFP expression was readily detectable after 24 hours in both tumor cell lines (Figure 4-3). This FACS analysis revealed that the percentage of tumor cells becoming GFP positive increased with increasing MOI (0-100) for both tumor cell lines. Maximal GFP expression in these ovarian tumor cells was observed 24 hours post-infection with rUF7 at an MOI of 100, with greater than 95% of both cell lines being GFP positive. Infection of these cell lines with rUF7 at MOIs ≥100 resulted in cell toxicity. rUF7 expression of GFP was much more efficient in the SAU cell line. For instance, ~90% of SAU cells scored positive for GFP expression following infection at an MOI of 1 compared to ~25% of SKA cells at this MOI.

GFP expression 24 hours following infection of SKA and SAU cells with rUF5 was < 10% in both cell lines (Figure 4-4). However, if these cell lines were coinfected with rUF5 and wild type adenovirus (MOI=3-5), GFP expression could be increased. Still even with helper virus infection an MOI of 100 resulted in only ~10% of SKA and ~75% of SAU cells expressing GFP.

No cellular toxicity was observed following infection with rUF5 at all MOIs tested.

Infection with Recombinant Adeno and AAV expressing NQO1 and GFP

To assess the ability of the recombinant adenovirus, rUF7-NQO1, and the recombinant AAV, rUF5-NQO1, to deliver the enzyme NQO1 and GFP in a dicistronic expression cassette, SKA and SAU cells were exposed to the range of MOIs previously employed for rUF7 and rUF5. GFP expression was readily detectable in both cell lines 24 hours post-infection with rUF7-NQO1 (Figure 4-5). However, GFP expression in the SKA cell line was dramatically reduced compared to that following a rUF7 infection. For example, rUF7-NQO1 only achieved a maximum of ~10% GFP positive cells with an MOI of 100 in the SKA cells compared to ~95% with rUF7 at this MOI (Figure 4-5 vs. 4-3). Like rUF7, rUF7-NQO1 was most efficient in the SAU cell line, although the efficiency of GFP expression with rUF7-NQO1 was reduced in this cell line as well. The maximum GFP expression in SAU cells required an MOI of 100 to achieve ~80% GFP positive cells. A photomicrograph of the SAU cell line expressing GFP after infection with rUF7-NQO1 is presented in Figure 4-6.

GFP expression in SKA and SAU cells was undetectable following infection with rUF5-NQO1 (data not shown). In addition, coinfection of SKA cells with wild type adenovirus (MOI=3-5) and rUF5-NQO1 (MOI=100) had no effect on GFP expression in this cell line. Maximum GFP expression in the SAU cell line was ~5% GFP positive cells at an MOI of 1000. Coinfection of

SAU cells with wildtype adenovirus (MOI=3-5) and rUF5-NQO1 (MOI=100) increased the number of GFP positive cells from 1 to 26%.

Having established the number of infectious units required for a significantly productive infection based on GFP expression in these cell lines, the ability of rUF7-NQO1 and rUF5-NQO1 to overexpress NQO1 in SKA and SAU cells also was examined. Infection of these cell lines with rUF7-NQO1 resulted in dose and time dependent increases in NQO1 levels (Figure 4-7). Again, this increase in recombinant adenovirus gene expression was more dramatic in the SAU cell line. rUF5-NQO1 failed to increase the level of NQO1 in these cell lines at these time points post infection. In order to determine if a longer time period post-infection was required for transcriptionally active recombinant AAV in these cell lines, NQO1 expression was determined 7 days post-infection (Figure 4-8). The longer incubation times post-infection did lead to an increase in NQO1 expression in both cell lines. However, this increase was significantly less than that following rUF7-NQO1 infection (Figure 4-7 vs. 4-8).

To investigate the potential to sensitize the SKA and SAU cell lines to aerobic treatment with EO9, both cell lines were infected with this recombinant virus, rUF7-NQO1, at an MOI of 10 and treated 24 hours later with a range of doses of EO9 (Figure 4-9). As an infection control, both tumor cell lines were infected with the recombinant adenovirus, rUF7, which expresses the reporter gene GFP but lacks NQO1. The results show that infection itself sensitizes SAU, but not SKA cells, to EO9 treatment (circles vs.

open squares). When compared at IC_{90} doses (dose of EO9 that kills 90% of tumor cells), SKA tumor cells infected with rUF7-NQO1 were ~2.3 fold more sensitive to EO9 (solid squares vs. solid circles). SAU cells were ~4.4-fold more sensitive to EO9 than uninfected cells (solid squares vs. solid circles) but this was to a large part the consequence of the ~2-fold enhancement in cell killing resulting solely from infection (open squares vs. solid circles).

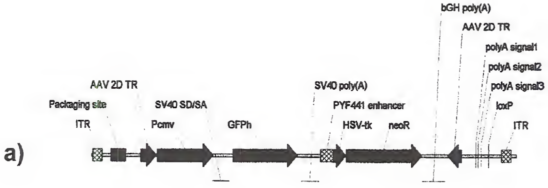
Discussion

In the present investigation recombinant adenovirus and AAV gene delivery systems that express GFP alone, and NQO1 and GFP together from a single CMV promoter were constructed. These vectors were found to be effective at delivering genes to the SKA and SAU ovarian tumor cell lines. In the case of recombinant adenovirus, high expression of GFP was noted in both SKA and SAU cell lines within 24 hours post-infection (Figure 4-3). The SAU cells in particular showed high expression at very low MOIs. For example, ~90% of SAU cells expressed GFP at an MOI of 1, whereas SKA cells required an MOI of 100 to achieve a similar level (Figure 4-3). This suggests that the SAU cell line may be more receptive to adenoviral infection than the SKA cell line. This conclusion is supported by experiments in which SKA and SAU cells were infected with the recombinant adenovirus rUF7-NQO1 and assayed for GFP (Figure 4-5) or NQO1 (Figure 4-7) expression. Again, transgene expression was more robust in the SAU cell line. Yet, from the results of Figure 4-5 it is also clear that GFP expression from the NQO1/GFP dicistronic cassette was impaired in the SAU cell line, and significantly diminished in the SKA cell line. One possible explanation for this observation is that the poliovirus type I IRES element possesses low efficiency at directing translation in the SAU cell line, and may be nonfunctional in the SKA cell line.

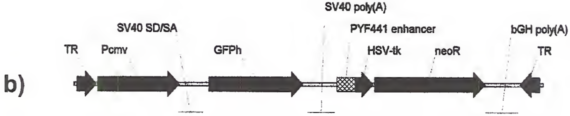
One of the goals of this study was to compare the efficiency of recombinant adeno- and adeno-associated viral gene delivery systems. Our results comparing infections with rUF7 and rUF5 demonstrate that recombinant AAV was much less effective than recombinant adenovirus in these ovarian tumor cell lines (Figure 4-4 vs. Figure 4-5). NQO1 could be expressed from this single stranded DNA virus but much higher MOIs and longer post-infection incubation times were needed (Figure 4-8). This result might be expected given the evidence that the rate limiting factor for efficient transduction of cells by recombinant AAV is the synthesis of the second DNA strand (Ferrari *et al.*, 1996). This process can be facilitated by coinfection of the recombinant AAV with wild type adenovirus, as well as by chemical and physical agents that have been shown to induce helper virus free DNA replication of wild type AAV. Our results suggest that this also may be the case for these ovarian tumor cell lines. When SKA and SAU tumor cells were co-infected with rUF5-NQO1 and wildtype adenovirus, both more rapid and higher levels of GFP (Figure 4-4) and NQO1 (Figure 4-8) expression were observed in both cell lines. These results suggest that recombinant adenoviral vectors may be the more effective vectors for employment in a VDEPT approach, as these vectors achieve higher levels of expression in a shorter period of time. While transgene expression from recombinant adenoviral vectors has been observed to be transient in most tissues (Zhang, 1999), this may not be a problem with a VDEPT approach as the cytotoxic agent targets the infected cell for killing. The ability of recombinant AAV to achieve more stable expression than recombinant adenovirus (Koeberl *et al*., 1999) suggests that this virus may be better suited for alternative gene therapy approaches to solid tumors in which long term expression of the transgene is important. The delivery to the tumor of immuno-modulating transgenes with the goal of enhancing the host immune response would be one setting where long term transgene expression would be more advantageous.

To examine whether the upregulation of the NQO1 enzyme following infection with a recombinant adenoviral vector expressing this transgene could lead to increased sensitivity of ovarian tumor cells to the quinone, EO9, SKA and SAU cells were infected with rUF7-NQO1 (MOI=10) and treated 24 hours later with a range of EO9 doses (Figure 4-9). Both cell lines normally express relatively low levels of this enzyme. The results showed that the delivery of NQO1 to these cells provided an effective means of sensitizing them to the aerobic killing effects of EO9. The increase in the aerobic sensitivity of the SAU cell line to EO9 following infection with rUF7-NQO1 was more dramatic than that seen in SKA cells; a finding that is in general agreement with the significantly higher levels of GFP (Figure 4-3) and NQO1 (Figure 4-7) achieved in the SAU line following a rUF7-NQO1 infection. However, it should be noted that infection itself renders SAU cells more susceptible to killing by EO9.

In conclusion, the present results have demonstrated that ovarian tumor cells are receptive to infection with both recombinant adenoviral and AAV gene delivery systems, although not necessarily to the same extent. Recombinant adenovirus was found to be capable of yielding higher levels of expression of the transgenes in a shorter period of time than recombinant AAV. Finally, the use of the NQO1/EO9 combination in a VDEPT approach to killing ovarian tumor cells demonstrated that increasing the levels of NQO1 could sensitize these cells to the bioreductive agent EO9. These findings suggest that constructs such as rUF7-NQO1 may have the utility in treating tumors inherently low in NQO1 expression or to sensitize those harboring mutant forms of NQO1. Moreover, these vectors may be used in combination with other bioreductive chemotherapeutic agents determined to be substrates for NQO1.



rUF7



rUF5

Figure 4-1. Expression cassettes of recombinant adenovirus (a) and recombinant AAV (b) that express GFP from a CMV promoter.

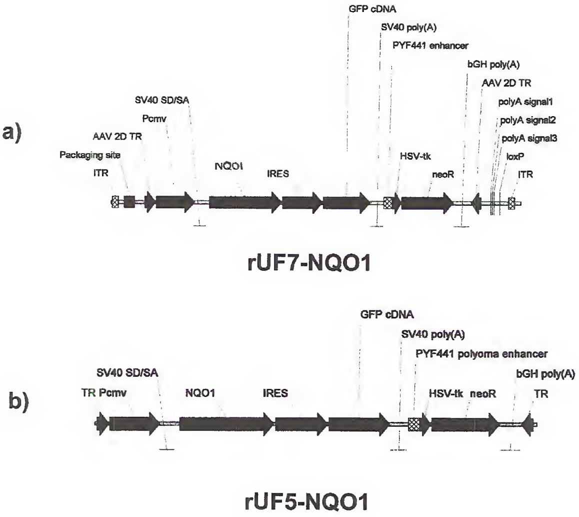


Figure 4-2. Expression cassettes of recombinant adenovirus (a) and recombinant AAV (b) that express NQO1 and GFP from a single CMV promoter through the use of an IRES element.

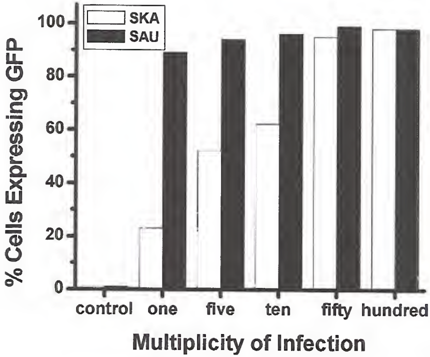


Figure 4-3. The effects of increasing MOI on the expression of GFP in SKA and SAU cells following infection with rUF7.

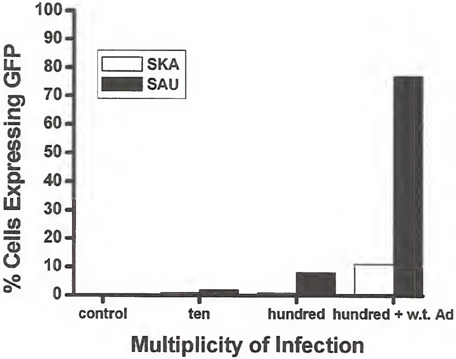


Figure 4-4. The effects of increasing MOI on the expression of GFP in SKA and SAU cells 24 hours post infection with rUF5 +/- wild type adenovirus.

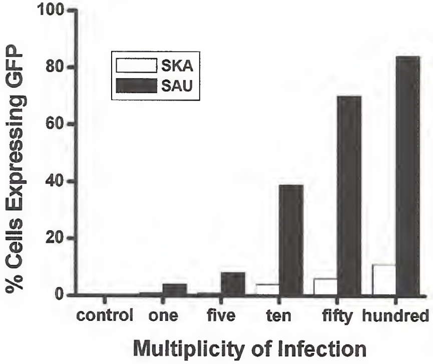


Figure 4-5. The effects of increasing MOI on the expression of GFP in SKA and SAU cells 24 hours post infection with rUF7-NQO1.

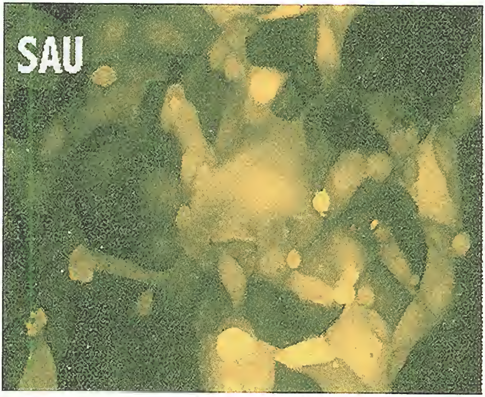


Figure 4-6. Photomicrograph of SAU cells 24 hours post infection with rUF7-NQO1 (MOI=100, 100X).

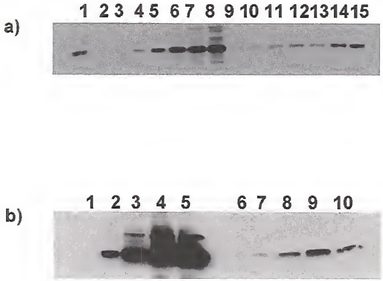


Figure 4-7. Western blot analysis of NQO1 levels in SKA and SAU cells following infection with rUF7-NQO1. a) Increasing MOI, 24 hour post infection. Lane 1: NQO1 standard; Lane 2: SAU, uninfected; Lane 3: SAU, rUF7 100; Lane 4: SAU, rUF7-NQO1 1; Lane 5: SAU, 5: Lane 6: SAU, 10; Lane 7: SAU, 50; Lane 8: SAU, 100; Lane 9: SKA, uninfected; Lane 10: SKA, rUF7 100; Lane 11: SKA, rUF7-NQO1 1; Lane 12: SKA, 5; Lane 13: SKA, 10; Lane 14: SKA, 50; Lane 15: SKA, 100. b) MOI=100, increasing time post transfection (hours). Lane 1: SAU, uninfected; Lane 2: SAU, 12; Lane 3: SAU, 24; Lane 4: SAU, 48; Lane 5: SAU, 72; Lane 6: SKA, uninfected; Lane 7: SKA, 12; Lane 8: SKA, 24; Lane 9: SKA, 48; Lane 10: SKA, 72.

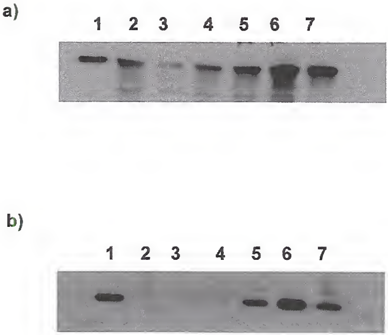


Figure 4-8. Western blot analysis of NQO1 level in SKA (a) and SAU (b) cells 7 days post infection with rUF5-NQO1. a) Lane 1: NQO1 standard; Lane 2: SKA uninfected; Lane 3: SKA, rUF5 500; Lane 4: SKA, rUF5-NQO1 10; Lane 5: SKA, 100; Lane 6: SKA, 1000; Lane 7: SKA, 100+wildtype adenovirus. b) Lane 1: NQO1 standard; Lane 2: SAU, uninfected; Lane 3: SAU, rUF5 500; Lane 4: SAU, rUF5-NQO1 10; Lane 5: SAU, 100; Lane 6: SAU, 1000; Lane 7: SAU, 100+wildtype adenovirus.

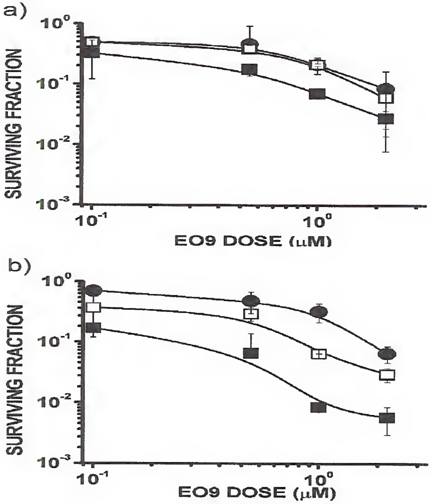


Figure 4-9. Virally directed enzyme prodrug therapy for SKA (a) and SAU (b) ovarian tumor cells using a recombinant adenovirus, rUF7-NQO1, in combination with the bioreductive chemotherapy agent, EO9. Closed circles: uninfected; Open squares: infected with rUF7; Closed squares: infected with rUF7-NQO1.

CHAPTER 5
INITIAL CHARACTERIZATION OF TRANSGENE EXPRESSION FROM rUF7 AND rUF7-NQO1 IN RODENT SOLID TUMORS

Introduction

Despite the proliferation of clinical protocols using gene therapy approaches for cancer (Roth and Cristiano, 1997), there are many aspects of gene transfer that are less than ideal. One of the most important areas of future research in gene therapy is vector design. The vector is critical for gene delivery and expression of the transgene, but all existing vectors have limitations. One major goal of improving gene therapy is to deliver the therapeutic genes only to the appropriate target cell *in vivo*. Improvements in the safety and effectiveness of gene therapy require the targeted delivery of the vector. One way in which the vector can be targeted to the appropriate cell type is through the route of administration. Examples of targeting by this physical method include *ex vivo* manipulation of target cells and/or localized application of the vector (Vile *et al.*, 1998).

Since adenovirus can transduce a large variety of human and rodent cell types, it is being explored as a vector to deliver transgenes to tumor cells *in vivo* (Robbins and Ghivizzani, 1998). Many solid tumor types lend themselves to direct injection of the vector. This type of localized delivery may improve the efficiency and selectivity of gene based strategies in cancer therapy.

Intratumoral injection of many viral gene delivery systems, including recombinant adenovirus, has been reported. This vector is currently being studied in a wide variety of genetic strategies for cancer therapy, including enzyme/prodrug therapy (Blackburn *et al.*, 1999). One important consideration in the employment of recombinant adenoviral vectors is that this virus rarely integrates into the host cell genome. Therefore, gene expression will be transient. While this is a major disadvantage in employing adenovirus to treat gene based metabolic disease it does not preclude its use in a cancer gene therapy strategy where the ultimate goal is to kill the transduced tumor cell. One major disadvantage of the use of recombinant adenoviral vectors is the existing host immune response to adenoviral proteins in the vector itself, or produced by the transduced cells, may increase the toxicity and/or limit the efficacy of any reintroduction of the vector.

In chapter 4 we describe the employment of recombinant adenoviral vectors to deliver GFP alone and in combination with the bioreductive enzyme, NQO1, to sensitize SKA and SAU ovarian tumor cells to the *in vitro* killing effects of the chemotherapy agent, EO9. Attempts at establishing human ovarian tumor xenografts with these two cell lines grown subcutaneously or intramuscularly in nude mice have thus far proved unsuccessful. However, a number of transplantable rodent tumor models are available that allow us to address the effectiveness of the recombinant adenoviral vectors, rUF7 and rUF7-NQO1, to deliver transgenes *in vivo*, while further investigations into the establishment of human ovarian xenografts are underway.

The purpose of the present investigation was to determine the effectiveness of rUF7 and rUF7-NQO1 for *in vivo* delivery of transgenes in the SCCVII and RIF-1 transplantable rodent tumor models.

Materials and Methods

Proviral Plasmid Construction and Recombinant Virus Production

The adenovirus proviral plasmid, pTRUF7, contains a CMV promoter controlling expression of GFP, a single lox P site for use in *Cre*-recombinase production of recombinant virus, the neomycin resistance gene under control of a fragment of the herpes simplex thymidine kinase promoter, and the entire expression cassette is flanked by adenovirus ITRs (Figure 4-1a). The adenoviral proviral plasmid, pTRUF7-NQO1, was constructed by inserting a dicistronic unit containing the human NQO1 cDNA and GFP cDNA separated by a poliovirus type 1 IRES element downstream of the CMV promoter (Figure 4-2a). Recombinant adenoviral vectors were produced using Cre-lox recombination (Hardy *et al.*, 1997).

Transplantable Tumor Models and Recombinant Adenovirus Injection

Approximately $1x10^5$ SCCVII adenocarcinoma and RIF-1 sarcoma cells were injected in the right flank of female C3H mice. Mice were anesthetized through inhalation of a 15% halothane in mineral oil solution prior to injection with recombinant viral vectors. Control animals were injected with similar volumes of saline. Tumors weighing ~0.5 grams (7-9 days post inoculation) were injected with $\sim 1x10^9$ recombinant adenoviral particles divided at two injection sites. Animals were killed and their tumors were removed 24 and 72 hours post injection with the recombinant viral vectors, rUF7 and rUF7-NQO1.

Tumor Sectioning and Fluorescent Microscopy

Freshly excised tumors were fixed in a 4% p-formaldehyde solution in PBS at 4° C for 48 hours. Fixed tumors then were placed in a 30% sucrose solution in PBS at 4° C for an additional 48-72 hours. The tumors then were removed from the sucrose solution, wrapped in aluminum foil, and placed at -80° C for 24 hours. Sections (~25-35 μm) were made of the frozen tumors on a cryostat and collected in PBS. These sections were mounted on glass slides and examined using fluorescent microscopy.

Preparation of Whole Cell Extracts and Western Blotting

Extracts were prepared using an extraction buffer containing 50 mM Tris (pH 8.0), 120 mM NaCl, 0.5% NP40 (v/v), 1 mM PMSF, aprotinin, and leupeptin (EBC buffer). Tumors were minced and homogenized in EBC buffer, incubated at room temperature on a rocking platform for 10 min, centrifuged at 1100 rpm for 10 min, and the supernatant was saved. Protein determinations (for gel loading) were made on the extracts by Bradford Assay (BIO-RAD). Fifty μg of total cellular protein from the extracts was added to each gel lane. A SDS-PAGE gel electrophoresis system (12% polyacrylamide) was used. Following gel run, the proteins were transferred from the gel to nitrocellulose membranes at 300 mA at 4°C for 3 hr. The membranes were washed with Tris-buffered saline (TBS) and blocked overnight with 5% dry milk in TBS solution at 4°C. The membranes were then incubated for 1 hr at room temperature with 5 ml of hybridoma supernatant containing an anti-NQO1 monoclonal antibody. The membranes were then washed with TBS for 30 min, and incubated with a 1:5000 dilution of a goat anti-mouse IgG conjugated to HRP (Promega, Madison, WI) for 30 min at room temperature. The blots were developed using chemiluminescence and the quantity of protein was determined using densitometry.

Results

Infection of Rodent Tumors with Recombinant Adeno Expressing GFP

The ability of recombinant adenovirus, rUF7, to infect and express the reporter gene GFP was examined in the SCCVII adenocarcinoma and RIF-1 sarcoma tumor models. Following infection with rUF7, GFP expression was detectable after 24 hours in both tumor models (Figure 5-1 and Figure 5-3). In addition, GFP expression was limited to a small subpopulation of tumor cell at the injection site these models. After 72 hours (Figure 5-2 and Figure 5-4), GFP expression remained detectable in both models; however, no significant increase in GFP expression was observed relative to 24 hours post injection (Figure 5-1 vs. Figure 5-2; Figure 5-3 vs. Figure 5-4).

Infection with Recombinant Adeno and AAV expressing NQO1 and GFP

To assess the ability of the recombinant adenovirus, rUF7-NQO1, to deliver the enzyme NQO1 and GFP in a dicistronic expression cassette, SCCVII and RIF-1 tumors were injected with the same dose of this recombinant adenovirus as was used for rUF7. No GFP expression was detected in either tumor model at 24 and 72 hours post infection. Likewise, no

NQO1 expression was detected by western blotting in tumor homogenates prepared 24 and 72 hours following infection with rUF7-NQO1.

Discussion

In the present investigation, recombinant adenovirus gene delivery systems that express GFP alone (rUF7), and NQO1 and GFP together (rUF7-NQO1) from a single CMV promoter were constructed and injected into rodent SCCVII and RIF-1 transplantable tumor models. rUF7 was found to be effective at delivering GFP to both models 24 and 72 hours post injection (Figure 5-1 to 5-4). However, because this expression was limited to the injection site in these tumors, a better approach for the employment of this vector may be to divide the total dose of injected virus into multiple injection sites, thus increasing the number of tumor cells encountering the vector.

As was the case with the ovarian tumor cell lines, it appears that the IRES element may be defective in these rodent tumor cell types because no GFP expression was observed following injection with rUF7-NQO1. The fact that no NQO1 was detected by western blotting following injection of this vector may be partly a result of the small subpopulation of tumor cells that were observed to express GFP following infection with rUF7 (Figure 5-1 to 5-4). In addition, whole tumor homogenates may further dilute the signal to levels undetectable by western blotting. An increase in the number of injection sites for rUF7-NQO1 may overcome this problem. Finally, analysis of NQO1 expression in tumor sections using immunohistochemical techniques may be a more sensitive approach to detecting overexpression of this enzyme following injection of tumors with rUF7-NQO1.

In summary, injection of SCCVII and RIF-1 tumors with rUF7 resulted in a small subpopulation of tumor cells, along the injection site, expressing GFP. Injection of rUF7-NQO1 into these tumor models resulted in no detectable NQO1 or GFP expression at the doses of recombinant adenovirus tested. Clearly, further studies are warranted to optimize recombinant adenoviral gene delivery in these models.

Future investigations into the relationship between the level of NQO1 and sensitivity to EO9 will proceed in the following manner. First, the establishment of human ovarian tumor xenografts from several of the other human ovarian tumor cell lines that possess a range of NQO1 levels is a way to address the NQO1/EO9 relationship *in vivo*. However, this approach will only allow an investigation into this relationship between different ovarian tumor cell lines. A more direct approach would be to employ gene therapy methods to sensitize those ovarian tumors deficient in NQO1 to the cytotoxicity of EO9 by first overexpressing the enzyme intratumorally. Future development of human ovarian tumor xenografts inherently low in NQO1 will focus on growing human ovarian tumor cells intraperitoneally in nude mice. This might be of particular interest because most ovarian cancer patients present with their disease confined to the intraperitoneal cavity. This localization would allow for the delivery of the vector systems and subsequent chemotherapy (enzyme/prodrug) by direct intraperitoneal injection. In addition, because this delivery route may achieve higher vector concentrations, intraperitoneal gene therapy may allow for higher levels of intratumoral transgene expression, while limiting delivery to normal tissues. Moreover, higher intratumoral prodrug concentrations may be achievable by this delivery route.

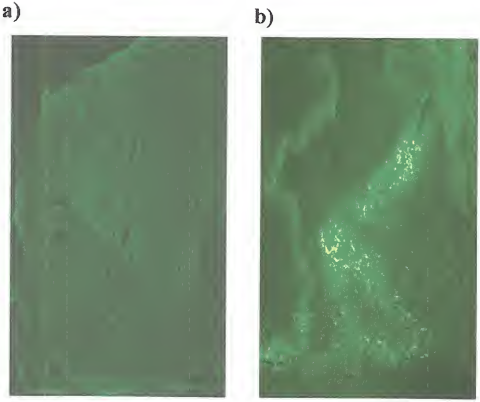


Figure 5-1. Analysis of GFP expression in SCCVII tumors 24 hours post injection with rUF7. a) uninfected; b) infected with rUF7.

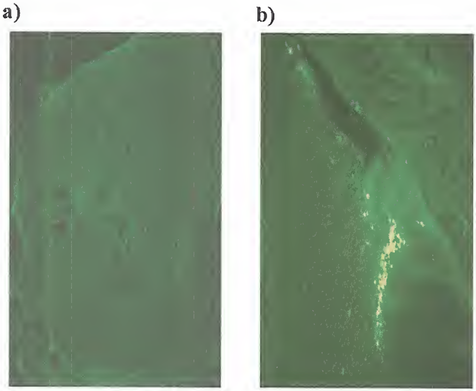


Figure 5-2. Analysis of GFP expression in SCCVII tumors 72 hours post injection with rUF7. a) uninfected; b) infected with rUF7.

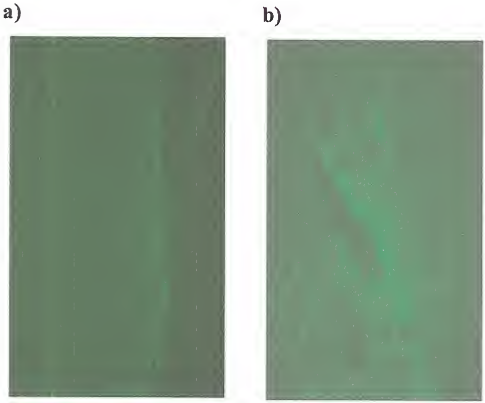


Figure 5-3. Analysis of GFP expression in RIF-1 tumors 24 hours post injection with rUF7. a) uninfected; b)infected with rUF7

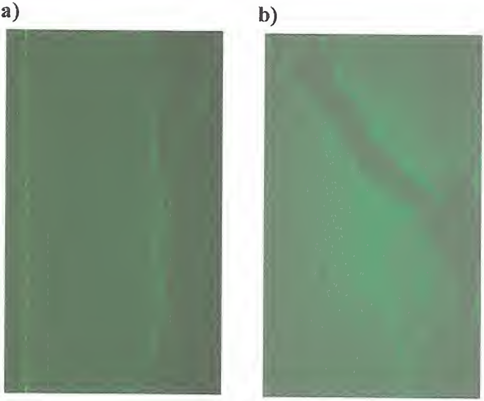


Figure 5-4. Analysis of GFP expression in RIF-1 tumors 72 hours post injection with rUF7. a) uninfected; b) infected with rUF7.

CHAPTER 6
CONCLUSIONS

Novel approaches for the employment of bioreductively activated drugs continues to be an area of active investigation in the field of experimental cancer therapy. Generally, the development of these compounds has focused on two design strategies. First, the anticancer agent may be designed to only kill hypoxic tumor cells, thus possessing high aerobic/hypoxic differentials. The cytotoxic effects of tirapazamine in this panel of human ovarian tumor cell lines are an example of this treatment strategy. In addition, this strategy exploits this aspect of solid tumors in order to achieve a level of tumor selectivity for the therapy, while targeting a population of tumor cells that is resistant to many conventional treatments. The employment of an agent such as tirapazamine would require additional treatments targeting the aerobic tumor cell population.

An alternative approach in bioreductive drug design is to develop compounds that are effective at killing aerobic tumor cells but kill hypoxic tumor cells more effectively. In contrast to an agent like tirapazamine, EO9 is a potent hypoxic cell cytotoxin that retains the ability to effectively kill aerobic tumor cells. In the context of bioreductive metabolism (Figure 6-1), a quinone antibiotic like EO9 could undergo a one electron reduction to the semiquinone generating a toxic species (red arrows). This pathway would predominate under hypoxic conditions because in the presence of oxygen the semiquinone would be reoxidized to the parent molecule. This semiquinone may then undergo a second one-electron reduction to a toxic hydroquinone. The results of this thesis demonstrate that the one electron reductase, CYPOR, might not be responsible for the hypoxic potency of EO9 in these ovarian tumor cells. Under aerobic conditions, two-electron reduction of a quinone to a toxic hydroquinone may occur (yellow arrows). Indeed, the results presented in this thesis demonstrate that the aerobic cytotoxicity of EO9 is related to the level of a particular enzyme in human ovarian tumor cells. It identifies a correlation between the level of the obligate two-electron reductase, NQO1, and ovarian tumor cell aerobic sensitivity to the bioreductive anticancer agent, EO9. Furthermore, these investigations demonstrate that this relationship can be exploited in a gene based enzyme directed prodrug approach to sensitizing those ovarian tumor cells inherently low in NQO1 to the killing effects of EO9. Finally, this work characterizes the levels of transgene expression obtained using recombinant adenoviral vectors as cancer gene delivery system *in vivo*.

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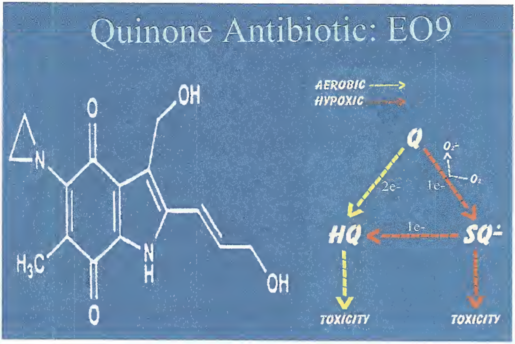

Quinone Antibiotic: EO9

Figure 6-1. Potential mechanisms of toxicity for the quinone antibiotic, EO9, under aerobic (yellow arrows) and hypoxic (red arrows) conditions. In the present investigations, a strong correlation between the obligate two-electron reductase, NQO1, and the efficacy of EO9 under aerobic conditions was demonstrated in a panel of human ovarian carcinoma cell lines. Furthermore, it was possible to improve the aerobic killing effects of EO9 using proviral vector plasmids and recombinant viral vectors to deliver NQO1 to ovarian tumor cells inherently low in this enzyme prior to administration of the bioreductive chemotherapy agent.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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